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SOME PRELIMINARY STUDIES ON MEAT FLAVOR[a, b, c]

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(Manuscript received May 31, 1958)

Although flavor is one of the major desirable attributes of meat, characterization and identification of the responsible components have not been accomplished. Crocker *(3)* reported that the flavor of raw meat is mainly confined to the juices, whereas cooked meat flavor appears to be due to chemical changes occurring in the fiber rather than in the juice. The flavor of cooked beef has been reported to be quite complicated chemically and to be mainly odor rather than taste *(3)*. Work with chicken indicates that the substances responsible for the chicken flavor can be extracted from the fiber by cold water *(6)*. Fat has been found to contribute little if any flavor to chicken broth, but does appear to contribute in some manner to the aroma *(2, 5)*.

This preliminary investigation was undertaken to characterize and ascertain the intensity of flavor in various beef and beef juice fractions.

EXPERIMENTAL

Source of meat. The meat used in all phases of this investigation consisted of the *Longissimus dorsi* muscle from a U. S. Good grade beef rib. Roasts were frozen and removed as needed for flavor studies.

Flavor threshold determinations. To determine the threshold at which the flavor could be detected a series of water dilutions were made and submitted to a panel of 3 to 5 judges. Each judge was asked to record the flask number in which he could first obtain a definite flavor. De-ionized distilled water was used to make all dilutions. Samples were at room temperature when tested. Whenever a filtrate was used, the solution was filtered through acid-washed Whatman 41-H filter paper, previously washed with de-ionized distilled water to remove the acid taste.

Values reported as the flavor threshold in this study are those reported by the majority of the judges. However, there was generally excellent agreement between different judges, and real discrepancies were apparent only on a few occasions.

Characterization of Flavor. Flavor was characterized by having the entire group of judges suggest descriptive terms until the group was satisfied that the taste sensations were adequately described. Raw meat, cooked meat, press fluid and press cake (both raw and cooked) were characterized and are reported.

Raw meat. The raw meat was made into a slurry (1 part meat to 2 parts of water) in a Waring blender and used for flavor threshold determinations. After the initial flavor threshold was ascertained, the slurry was filtered and the filtrate collected.

Raw meat filtrate. After the maximum dilution at which flavor could be detected was determined, the filtrate was heated for one hour over a steam bath at 93° C. The original filtrate was cooled and the flavor threshold was again determined to see if heating had any effect on intensity of flavor. The previously heated filtrate was again filtered to remove the slight precipitate that formed on heating, and to determine whether the flavor was concentrated in the precipitate or the aqueous portion.

Raw meat residue. A portion of the residue remaining on the filter paper after the initial filtration was tasted and the flavor characterized. The remaining residue was re-

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[a] Journal Article *2268* Michigan Agricultural Experiment Station, East Lansing.

[b] Presented at Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

[c] A portion of the research completed for the Ph.D. thesis by the senior author.

suspended in water, heated over a steam bath at 93° C. for one half hour, cooled, and the flavor characterized again to ascertain the influence of heating upon the raw meat fibers.

Cooked meat. The meat was roasted to an internal temperature of 74° C. in an electric oven held at 149° C. A meat-water slurry was prepared in the same manner as with the raw meat; the same procedure was followed as that used for studying the slurry, filtrate, and residue of the raw meat.

Press fluid from raw beef. The meat juice was extracted from the fibers with a Carver Laboratory Press. Three grams of shredded filter paper were mixed with every 100 g. of coarsely ground meat before pressing. Each 100-g. sample of meat was pressed for 10 minutes at a pressure of 6,000 lbs. psi., after the pressure was attained gradually during a 5-minute period, and the volume of press fluid was recorded. The flavor was characterized by the panel and the flavor threshold was determined by dilution. The remainder of the press fluid was heated for one hour over a water bath at 93° C., cooled, and the flavor threshold again determined to ascertain the effect of heating on the flavor of press fluid from raw beef. The material, which had coagulated upon heating, was removed by filtering, and the threshold determination made upon the filtrate.

Press cake from raw beef. The press cake from raw beef was tasted by the panel and its flavor characterized. Then a 1-3 water slurry was prepared and simmered at 93° C. for one hour. The slurry was cooled to room temperature, tasted by panel members and the flavor was characterized. The slurry was then filtered to remove the fibers and fat particles and the filtrate was tasted.

Press fluid from cooked beef. The meat was prepared by roasting in the manner previously described. Press fluid was collected by the procedure used for the raw beef, and flavor threshold determinations were made both before and after filtering.

Press cake from cooked beef. Two parts of water by weight were added to one part of press cake. The suspension was heated the same way as the raw press cake, tasted, filtered and the filtrate tasted.

Leaching studies on meat flavor. *Raw Beef.* Seventy grams of raw beef were cut into 1-inch cubes, covered with 300 g. of de-ionized distilled water and placed in a refrigerator held at approximately 4° C. At 24-hour intervals the beaker was removed and the meat was squeezed by hand. Leachings were collected and used for further studies. The meat was again covered with 300 ml. of previously cooled water and returned to the refrigerator. The flavor threshold was determined for the leachings and the remainder of the leachings was heated over a 93° C. steam bath for one hour. The heated leachings were then cooled and threshold determinations made.

Cooked beef. A beef roast was roasted as described previously. The meat was cut into cubes and treated the same as the raw meat except that the leachings were not simmered.

Chemical Analysis: Dry matter and ether extract were determined for the raw beef roast, the cooked beef roast, raw press fluid, cooked press fluid, raw press cake and cooked press cake with modification *(4)* of the procedure described by Benne, Van Hall and Pearson *(1)*. In addition, pH readings were taken at each step throughout the investigation.

RESULTS AND DISCUSSION

Studies on Raw Beef

Characterization of flavor. Raw beef was characterized as blood-like, slightly salty, and somewhat pleasant. When the taster held his nostrils, a slightly astringent effect was noted but no other flavor was perceptible. On releasing the nostrils, a flood of flavor filled the mouth.

Flavor threshold. Figure 1 shows the flavor threshold determinations of the various raw beef fractions studied. The maximum dilution at which the raw beef could be detected was 1-50, and after filtering it could still be detected at 1-50. Thus, it is apparent that the raw beef flavor was primarily located in the juice, which is in agreement with Crocker's results *(3)*. After simmering the raw beef filtrate, the flavor threshold was found to be 1-70 and remained the same after filtering. Consequently, the flavor was intensified on cooking the juice as shown by the change in flavor threshold. In addition,

it was shown that the raw juice developed a cooked meat flavor on heating, which shows that at least part of the cooked meat flavor is present in the juices in contrast to Crocker's *(3)* conclusion that the fibers are primarily responsible for cooked meat flavor.

The raw residue remaining after filtration had a chalky, bitter, and unpleasant flavor. Heating the water-residue mixture resulted in development of a pleasant, somewhat bland, slightly meaty flavored solution. Filtering the mixture did not change the flavor characteristics of the filtrate. Thus, it is evident that the meaty flavor is water soluble and was largely removed in the aqueous portion of the original filtrate from the meat-water slurry.

Studies on Cooked Beef

Characterization of flavor. Cooked beef was characterized as being pleasant and meaty. When the taster held his nostrils, no flavor was detectable, but on releasing the nostrils, a flood of pleasant meaty flavor filled the mouth. Thus, the flavor of cooked beef appeared to be closely allied with the sense of smell.

Flavor threshold. Flavor threshold values for different roast beef fractions are presented in Figure 1. The original roast beef could be detected at

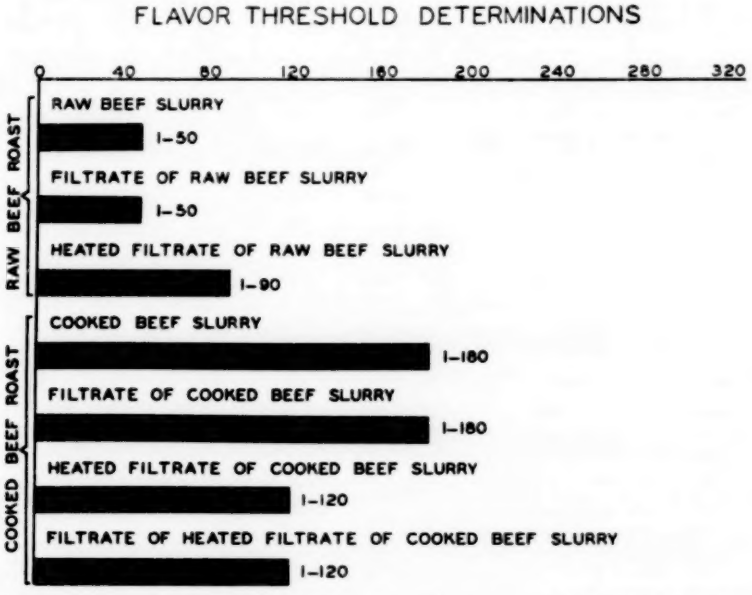

Figure 1. Flavor threshold determinations for various raw and cooked beef fractions.

1-180, and filtering did not change the threshold. It is evident that the flavor components are water soluble and are not bound to the fibers. When the water filtrate was heated over a steam bath, the flavor threshold was lowered to

1-120, which did not change on filtering off the coagulable portion. The drop in the flavor threshold is probably due to a loss of the volatile flavor constituents during heating. Since the amount of fat-free dry matter (Table 1) is greater in the heated filtrate than in the original filtrate, it is obvious that the flavor changes observed are not a function of dry matter.

The difference in flavor thresholds between raw and cooked meat indicates that cooking develops or intensifies the meat flavor. Examination of the differences in fat-free dry matter (Table 1) between raw and cooked beef at the flavor threshold reveals that the cooked beef can be detected at a greater dilution of fat-free dry matter. Since the flavor was not nearly as apparent on heating the raw fibers in water, it is postulated that heating the juices and fibers together may intensify or enhance the development of flavor.

TABLE 1

Flavor thresholds and chemical determinations on various meat fractions [1]

	Flavor threshold	pH	Dry matter	Ether extract	Fat free day matter
			%	%	%
Raw beef roast	—	—	29.87	8.63	21.24
Raw beef slurry	1-50	5.50	.60	.17	.42
Filtrate of raw beef slurry	1-50	5.50	.60	.17	.42
Heated filtrate of raw beef slurry	1-90	5.72	.33	.10	.24
Cooked beef roast	—	—	41.50	15.19	26.31
Cooked beef slurry	1-180	6.10	.23	.08	.15
Filtrate of cooked beef slurry	1-180	6.10	.23	.08	.15
Heated filtrate of cooked beef slurry	1-120	6.10	.35	.13	.22
Filtrate of heated filtrate of cooked beef slurry	1-120	6.10	.35	.13	.22
Raw press fluid	—	—	14.02	.21	13.81
Raw press fluid	1-70	5.34	.20	.003	.20
Heated raw press fluid	1-260	5.70	.05	.0008	.05
Filtrate of heated raw press fluid	1-260	5.70	.05	.0008	.05
Raw pressed fiber	—	5.44	59.02	6.81	52.21
Heated raw pressed fiber	—	5.70	—	—	—
Cooked beef press fluid	—	—	11.62	0.31	11.31
Cooked beef press fluid	1-300	6.00	.03	.001	.038
Filtrate of cooked beef press fluid	1-300	6.00	.03	.001	.038
Cooked pressed fiber		5.92	67.35	7.45	59.90

[1] All fat, dry matter and fat-free dry matter percentages are based on the original composition of each roast due to difficulty in obtaining suitable methods for such high dilutions. Thus, no corrections are made for material removed by filtering.

The cooked meat residue remaining after the first filtration had a mild, pleasant roast beef flavor. On heating a water suspension of the residue, it developed a somewhat stronger roast beef flavor. Thus, indications are that the flavor is retained more tenaciously by the fibers after heating, which is in partial agreement with Crocker *(3)*. On filtering the water-residue suspension, the filtrate was strong and slightly bitter, whereas the remaining residue was bland, flat, and slightly beefy.

Press Fluid Studies

Characterization. Undiluted raw press fluid was characterized as being strong, unpleasant and visceral-like. The flavor of the press fluid from cooked beef was less objectionable than the raw, but it was rather strong and somewhat visceral or animal-like in taste. The flavor of both the raw and cooked press fluid appeared to be predominately odor.

Flavor threshold. The raw press fluid had a flavor threshold of 1-70, which increased to 1-260 upon heating. Removal of the fat and coagulated material did not alter the level of detection. Furthermore, the flavor of the filtrate was very strong but meaty. Thus, the flavor components in beef could be pressed from the fiber. Furthermore, the flavor was intensified upon heating the filtrate, which shows that cooking develops or enhances the flavor of the press fluid. It is interesting that the fat-free dry matter at the detection threshold of heated press fluid was only one-fourth as high as it was at the flavor threshold for the raw press fluid (Table 1). It is therefore evident again that flavor is not a function of dry matter.

The flavor threshold for press fluid from roast beef was found to be 1-300 and was not altered by filtration. Cooking prior to pressing appeared to intensify the flavor of the press fluid, which again indicates cooking of the fibers and juice together resulted in greater flavor development.

On heating the raw press cake in water, a pleasant beefy flavor was developed. Although a fatty film was present on the surface after heating, the

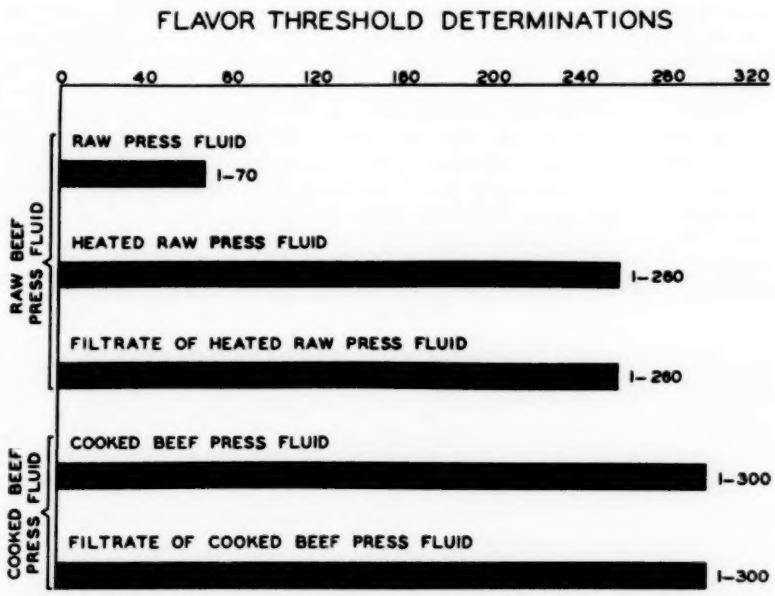

Figure 2. Flavor threshold determinations for various raw and cooked press fluid fractions.

flavor was still present in the filtrate to about the same extent. Hence, flavor did not appear to be associated with the fatty portion. Examination of the data in Table 1 on percentage fat at different flavor thresholds also verifies the fact that intensity of flavor and fat content are not related, which is in agreement with work on poultry *(2, 5, 6)*.

Chemical Components

It has been pointed out previously that neither fat content nor fat-free dry matter are responsible for meat flavor. Although cooking brought about an increase in pH, the change in pH certainly does not account for most of the flavor changes since it becomes more difficult to identify pH changes as neutrality is approached.

Leaching Studies

The flavor of raw meat leachings were characterized as being slightly blood-like and bland; the flavor of the leachings from cooked meat was meaty and slightly astringent. The raw meat was devoid of flavor after leaching for 3 days and the cooked meat after 5 days.

Flavor thresholds of the raw leachings were 1-50, 1-20 and 1-5 at the end of the first, second and third 24-hour periods of leaching, respectively (Figure 2). The detection threshold was found to increase upon heating the leachings to 1-100 for first period, 1-60 for the second and 1-30 for the third 24 hour period. With the cooked meat leachings, the threshold was 1-40 after the first period, 1-15 for the second, 1-10 for the third and 1-5 after both the fourth and fifth 24 hour leaching periods (Figure 2). Leaching could not be carried out longer with either the cooked or raw series because spoilage ensued.

Results of the leaching studies show the flavor of beef can be leached out of both the cooked and raw beef, although the cooked beef fibers appear to bind the flavor components more tenaciously. The binding effect is evident from the lower initial flavor threshold of the cooked leachings and the longer period of time over which flavor could be leached from the cooked meat. The leaching of flavor from beef is in agreement with the results of Pippen, Campbell, and Streiter *(6)* for poultry.

SUMMARY

This investigation was undertaken to characterize and ascertain the intensity of flavor in various meat and meat juice fractions. A dilution technique of ascertaining the detection threshold with a panel was used for the experiment. Raw and cooked beef water slurries, raw and cooked press fluid and filter cake, and raw and cooked meat water leachings with the various filtrates, both before and after heating, were used to study flavor changes.

The character of the flavor varied greatly between the raw and cooked fractions, yet upon heating the raw fractions the flavor appeared to differ only in intensity. It was evident that press fluid had a highly concentrated flavor. Results showed the flavor constituents were largely water soluble in both the cooked and raw fractions. However, cooking prior to extraction increased the flavor threshold, indicating that full flavor development may be

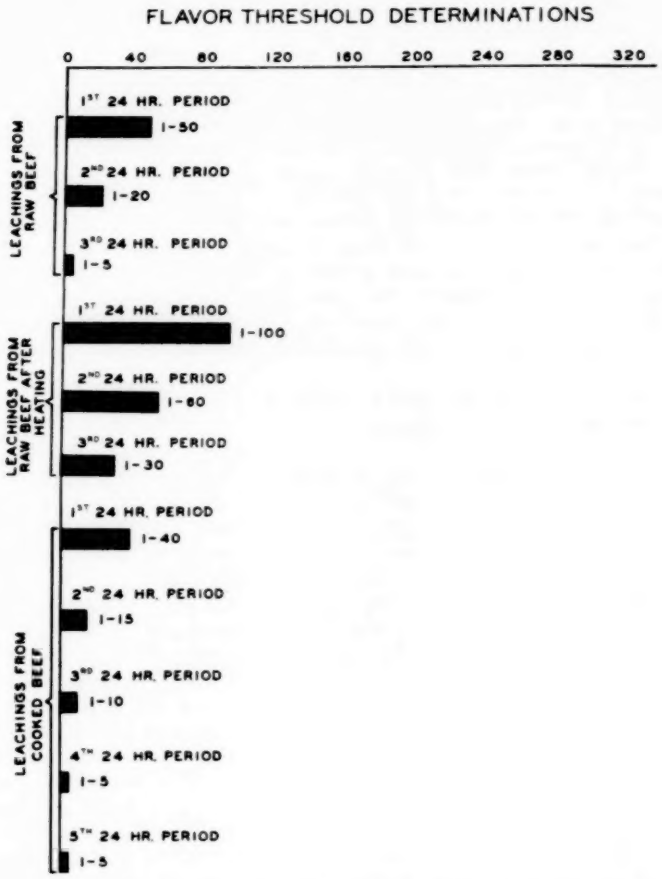

Figure 3. Flavor threshold determinations for leachings from raw and cooked beef roasts.

due to heating of the juice and fibers together. Leaching of the meat with water resulted in a complete loss of flavor with both cooked and raw meat, but the cooked meat maintained its flavor over a longer period of time.

Comparisons of flavor threshold with gross chemical analysis showed that neither fat content nor fat-free dry matter were responsible for differences in flavor. Although changes in pH occurred on heating the various meat and meat extracts, such changes did not appear to be responsible for flavor differences.

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EFFECT OF CERTAIN CHEMICALS IN WATER ON THE FLAVOR OF BREWED COFFEE*

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(Manuscript received March 28, 1958)

Organoleptic studies of odor-producing impurities which might impair the taste of water supplies and coffee brewed therefrom have been conducted and taste thresholds of six odor-producing chemicals and four non-volatile compounds used in water treatment have been estimated. These experiments supplement earlier work by Lockhart, Tucker, and Merritt *(2)* which was concerned with threshold levels of inorganic ions normally present in water supplies.

Ammonia, chlorine, hydrogen sulfide, phenol and the ortho- and para-chlorophenols were selected as typical examples of odor-producing impurities found in some municipal water supplies.

It was assumed that coffee-making equipment and china would retain traces, at least, of chemicals used for cleansing and that coffee brew might contain water softening compounds in appreciable amounts. Disodium dihydrogen versenate, commonly used as a sequestering agent, and Calgon, were selected as representative water-softeners. Trisodium phosphate, a heavy duty cleanser, and Tide, an all-purpose detergent, were also included in this phase of the experiment. Threshold levels were determined for the selected compounds in water and in coffee brew made from water solutions of the respective chemicals. Effect of these chemicals on the flavor of the brewed coffee was also noted.

Solutions of the chemicals were prepared with redistilled water in terms of parts per million. Residual chlorine was determined in water heated by the method for preparation of standard coffee brew *(2)*. Threshold values were reported in terms of residual chlorine. Variable losses of hydrogen sulfide made reliable estimates of thresholds in treated coffee brew unfeasible. The negligible losses of ortho- and parachlorophenol during brewing were confirmed by the use of standard curves obtained with a Beckman Model DU Quartz Spectrophotometer.

Concentrations chosen for triangle tests were such that the odd sample would be identified by more than 35% but less than 100% of the judges.

The coffee used was one selected from several as an acceptable brand. It was a Silex-grind type and vacuum-packed in one-pound cans. The coffee brew was prepared in Silex glass coffee makers by the method previously used *(2)*. Controls were brewed with redistilled water and treated samples were brewed with the solutions described above.

EXPERIMENTAL

Method. Organoleptic panels of 21-22 experienced judges participated in difference tests of the triangle type. Since panel members were selected from among the depart-

* Contribution No. 328 from the Department of Food Technology, Massachusetts Institute of Technology, Cambridge, Mass.

mental staff and graduate students, membership varied day by day. Panel tests were conducted only at periods about 2 hours before and after the noon hour.

Triangle tests were used both for threshold concentrations in water and for detectable levels in coffee brew. Samples were served in 25-ml. portions in coded 150-ml. beakers. Combinations were randomized and judges were asked only to identify the odd sample. In the case of coffee brew only the judges were asked to score the samples on the hedonic scale.

Data for the determination of taste thresholds of additives in water are given in Table

TABLE 1

Data for the measurement of taste thresholds of chemicals in water solution

Chemical	Conc. ppm	Separations		Percentages	
		Total	Correct	Correct	Above chance
Ammonia	26	21	13	61.9	42.9
	52	21	15	71.4	57.0
	104	21	18	85.7	78.6
Calgon (sodium hexametaphosphate)	150	21	12	57.1	35.7
	200	21	14	66.7	50.1
	250	21	16	76.2	64.2
Chlorine	0.25	22	10	45.5	18.3
	2.6	21	13	61.9	42.9
	5.3	21	14	66.7	50.1
O-Chlorophenol	0.002	21	11	52.4	28.5
	0.004	21	13	61.9	42.9
	0.05	21	19	90.5	85.8
	0.1	21	18	85.7	78.6
P-Chlorophenol	0.5	21	10	47.6	21.5
	1.0	21	12	57.1	35.7
	1.5	21	20	95.2	92.7
P-Chlorophenol (in tap water)	1.0	21	11	52.4	28.5
	1.5	21	15	71.4	57.0
Disodium dihydrogen versenate	75	22	10	47.6	21.5
	150	21	16	76.2	64.2
	250	21	16	76.2	64.2
	350	21	16	76.2	64.2
Hydrogen sulfide	0.010	22	10	45.5	18.3
	0.027	21	7	33.3	0
	0.051	21	18	85.7	78.6
	0.056	21	17	81.0	71.7
Phenol	14	21	9	42.9	14.4
	35	21	11	52.4	28.5
	89	21	16	76.2	64.2
Tide (all purpose detergent)	10	21	11	52.4	28.5
	14	21	17	81.0	71.4
Trisodium phosphate	200	21	13	61.9	42.9
	225	21	9	42.9	14.4
	250	21	15	71.4	57.0

TABLE 2

Data relating to the effect of chemicals on the flavor of coffee brew

Chemical	Conc. ppm	Separations		Hedonic scores	
		Total	Correct	Control	Test
Ammonia	104	21	12	4.8	5.2
Calgon (sodium hexametaphosphate)	700	21	12	4.7	5.8
	900	21	13	6.6	5.4
	1000	21	15	5.6	4.4
Chlorine	29	21	8	5.8	5.0
	82	21	11	5.6	3.9
	114	21	15	5.6	2.5
	159	21	17	5.8	2.4
O-Chlorophenol	0.0005	21	8	6.6	5.2
	0.001	21	15	5.4	4.9
	0.005	21	15	5.6	4.3
	0.01	21	14	5.4	3.5
	3.0	21	20	6.5	2.9
P-Chlorophenol	1.0	21	8	—	—
	2.0	21	11	—	—
	3.0	21	12	5.3	4.1
	4.0	21	15	5.9	3.5
	5.0	21	16	6.3	4.0
P-Chlorophenol (in tap water)	1.0	21	11	6.3	4.9
	2.0	21	18	6.6	3.0
	3.0	21	16	6.7	3.1
	5.0	21	19	6.9	4.3
Disodium dihydrogen versenate	600	21	8	5.8	5.4
	700	21	12	6.3	4.9
	800	21	10	5.2	3.8
	900	21	16	5.6	4.4
Hydrogen sulfide	0.056	21	8	6.1	6.0
	0.118	21	9	5.9	5.1
	0.491	21	5	5.1	4.4
	3.34	21	6	6.0	7.1
	29.85	21	8	4.9	3.9
Phenol	14	21	8	5.4	5.4
	28	21	7	5.4	4.9
	42	21	10	5.3	5.3
	56	21	8	4.8	5.1
	112	21	15	5.3	4.0
Tide (all purpose detergent)	32	21	9	5.3	4.9
	40	21	14	5.9	5.1
	40	21	11	6.1	5.5
	45	21	12	6.5	5.8
	50	21	8	4.6	4.5
	75	21	10	5.3	5.4
	150	21	17	5.7	4.5
Trisodium phosphate	1000	21	9	5.0	5.5
	1400	21	14	4.8	5.0
	1700	21	14	5.5	5.5

TABLE 3

Threshold concentrations of selected chemical impurities in water and coffee

Compound	Threshold in water ppm	Threshold in coffee brew ppm
Odor-producing chemicals		
Ammonia	34.0	104.0 [1]
Chlorine	5.2	108.0
Chlorophenols		
ortho-	0.006	0.001
para-	0.9	3.60
para-	1.35 [3]	1.33 [3]
Hydrogen sulfide	0.05	0.12 [2]
Phenol	60.0	105.0
Detergents; water softeners		
Calgon	200.0	935.0
Disodium dihydrogen versenate	120.0	825.0
Tide	12.0	100.0
Trisodium phosphate	225.0	1550.0

[1] Color change prevented use of higher concentrations.
[2] Losses by volatilization made higher levels difficult to measure.
[3] Tap water substituted for redistilled water in water solution and coffee brew.

1. Results of organoleptic tests on coffee appear in Table 2, and threshold concentrations for both water and coffee are summarized in Table 3.

When 50% above chance of the judges separated the samples correctly in a given triangle test, the concentration of additive used in that test was taken as the threshold level.

DISCUSSION

The threshold of ammonia in redistilled water was found to be 34 p.p.m. Free ammonia is seldom reported by water departments. Amounts of 0.023 p.p.m. and 0.047 p.p.m. in water from 2 sources appeared in an average report for 1952 from New York City's Bureau of Water Supply (private communication).

At a concentration of 104 p.p.m. in coffee brew, ammonia-treated samples were correctly separated from the control by only 23.8% above chance of the judges on a panel. Efforts to obtain a threshold concentration, at 50% above chance, were thwarted by visible color changes in the coffee.

Residual chlorine seldom appears in municipal water reports. A typical analysis from Miami, Florida, in 1950, listed 0.5 p.p.m. of chlorine at the plant and 0.1 p.p.m. in the distribution system (private communication, Department of Water and Sewers). Both concentrations are far below the thresholds in Table 3.

Low thresholds of hydrogen sulfide were found, both in redistilled water and in brewed coffee (Table 3). In the Miami report, quoted above, the hydrogen sulfide content of raw water was 0.5 p.p.m., whereas that of treated water was zero. In general, this offensive compound does not appear in analyses of public water supplies.

Phenolic compounds in potable waters entering interstate commerce are limited to 0.001 p.p.m. *(3)*. Table 3 shows the thresholds for phenol and, with one exception, thresholds for ortho- and parachlorophenols, to be somewhat higher than the foregoing tolerance. When a water supply is contaminated with industrial wastes, presence of chlorophenols will create problems because of their persistent nature. Removal of ortho- and parachlorophenol from a stream is said to require a highly specialized organism, and a much longer time for biological oxidation is needed than is required for removing phenols and cresols *(4)*.

Water softeners and cleansing compounds were tested in both water and coffee brew to determine whether flavor thresholds were low and in the range of concentrations which might conceivably remain in softened water or in kitchen equipment cleansed with such compounds. If the several thresholds were found to be high, it could be assumed that the consumer of coffee would be unaware of trace contaminants.

Table 3 shows the thresholds found in water and coffee brew for Calgon, disodium dihydrogen versenate, Tide, and trisodium phosphate. The detectable concentrations in all cases are probably higher than the residual amounts of the 4 compounds found in water and in coffee-making equipment. The threshold level of Tide is a possible exception. It is low enough to indicate that china, glass, and other containers should be rinsed thoroughly after being cleaned.

When the flavor of control and treated coffee brews was scored by the panel on a hedonic scale, some interesting results were observed. Scores were variable, but were not appreciably lower for maximum concentrations than for minimum concentrations of the respective additives (Table 2). Exceptions were found in the cases of ortho-chlorophenol, hydrogen sulfide, and phenol.

SUMMARY

Taste thresholds have been estimated in redistilled water and coffee brew for six odor-producing chemicals and for four non-volatile compounds used in water treatment. Threshold concentrations were generally found to be well above amounts of the chemicals known or assumed to be found in public water supplies. Taste thresholds of additives measured in coffee were usually higher than the respective thresholds found in water solution.

Acknowledgment

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DISCERNMENT OF TASTE SUBSTANCES AS AFFECTED BY SOLVENT MEDIUM[a]

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The aspect of taste-test research reported herewith deals with the effect of a lipid medium as contrasted with a water medium on taste perception. The purpose was to determine the part that fatty substances may play in carrying taste, and to contribute information on the tissue penetrability of lipoidal material.

Evidence has been presented to the effect that lipoid solubility contributes to taste perception. In discussing the anomalous sourness of weak organic acids, Moncrieff *(4)* points out that acetic acid, for example, is a much better solvent for fatty substances than mineral acids.

Taylor, Farthing and Berman *(6)* found that the introduction of polar groups to organic acids reduced the penetrating power, while increase in length of the carbon chain facilitated penetration.

Parker *(5)* states that the sweet taste and the bitter taste are primarily associated with organic compounds. The sweet taste and the bitter taste are excited both by ions and by molecules. The sweet taste, notably, is produced by a variety of compounds which are not ionized. Loginov, as reported by Moncrieff *(4)* states that in a homologous series of sweetening substances, an optimum sweetness for a certain size of molecule and solubility is always found. He states further that the sweetness of aromatic sweetening substances such as saccharin and dulcin can be correlated with their water solubility as well as with other physical and chemical properties. Yet the water solubility of saccharin is very low.

Bitter taste is chiefly associated with alkaloids and certain glucosides, but is given by a variety of other compounds, including the large group typified by phenylthiocarbamide, the bile salts, picric acid, and magnesium, ammonium and calcium salts, the cations of these inorganic salts being the effective agents *(4)*.

The bitter taste is widely distributed in nature, being present in nuts, fruits, vegetables, cereals and many other foods *(1)*.

According to Parker *(5)*, the stimulus for taste is an aqueous solution. Materials insoluble in water are tasteless. Yet if water solubility is accompanied with lipoid solubility more general access to the receptors of taste is possible with correspondingly more acute sensation *(4)*.

If lipoic solubility increases penetration of tissues, it should follow that taste substances having the property of solubility in both aqueous and organic media, when dissolved in a lipid medium should be especially easy to detect.

PROCEDURE

In order to secure evidence for the perceptibility of taste substances in a lipid medium, it was necessary to compare the perceptibility of taste of the same substance in water

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solution. Taste substances suitable for study were necessarily soluble both in oil and water. Substances that are available commercially, that are suitable for taste testing, and that are soluble in both oil and water are few.

Caffeine, quinine, and saccharin were selected for study. The solubility of these substances is shown in Table I *(2)*. Although only slightly soluble in some media, the amount of substance that could be dissolved was ample for testing thresholds of taste both in oil and water medium.

Solutions were prepared using oil as the solvent for one series and water as the solvent for the other series. The oil selected was highly refined peanut oil, having a minimum of taste or flavor. Identical concentrations of a given substance were used in these parallel series. The concentrations used are shown in Table 2.

TABLE 1

Solubility of substances used for testing the effect of solvent medium on taste discernment. (Ref. 2)

Substance	Mol. Wt.	Solubility Grams per 100 ml.				
		Water	Alcohol	Ether	Chloroform	Acetone Benzene
Caffeine ($C_8H_{10}O_2N_4 \cdot H_2O$)	212.21	1.35 [16] 45.5 [65]	2.3 [16] 85%	0.044 [16]	14.2	s
Quinine ($C_{20}H_{24}O_2N_2 \cdot 3H_2O$)	378.46	0.064	1.54	73.8	s	s
Saccharin ($C_7H_5NO_3S$)	183.18	0.43	3.1	sl.s	sl.s	s

TABLE 2

Concentrations of taste substances in test solutions

Solution number	Substance					
	Saccharin		Quinine		Caffeine	
	Molarity	g/1000 ml	Molarity	g/1000 ml	Molarity	g/1000 ml
1	.0000188	.0034	.0000062	.0023	.00025	.0531
2	.000025	.0046	.0000125	.0047	.0005	.1061
3	.00005	.0092	.000025	.0095	.001	.2122
4	.000075	.0137	.00005	.0189	.0015	.3183
5	.0001	.0183	.0001	.0378	.002	.4244
6	.000125	.0229	.00015	.0568	.0025	.5305

Since the texture of a food may affect its taste *(3)*, the viscosity of the oil was a problem. Obviously, the viscosity could not be reduced to that of water. A third series was therefore included consisting of water solutions plus methylcellulose. The viscosity of these solutions corresponded to that of the oil. The cellulose did not contribute flavor or taste to the solutions. The viscosity of the oil was 115 c.p. at 25° C. Thirty-five tablets of methylcellulose, each 0.5 g., were added to 1000 ml. water to obtain a viscosity equal to that of the oil.

Each of the 6 concentrations was coded and randomized within each of 3 replications. The water and oil replications were alternated, to help avoid fatigue of the judges when tasting oil solutions. The judges were informed as to whether they were tasting for bitter or sweet. They compared each sample with a control which contained no added taste substance. When the unknown tasted sweet or bitter as compared with the control, the judge indicated this on a score card. Threshold values were of interest; hence, the response to relative sweetness or bitterness was not determined. Comparison with the control, rather than use of a triangle test was chosen, since both the bitter and sweet substances used in this experiment, had a lingering after-taste. This would have made an accurate selection of the "different" sample in a triangle test very difficult.

RESULTS AND CONCLUSIONS

Six judges completed the series of tests of 3 substances each dissolved in oil, water, and water plus cellulose. For ease of comparison, the data are plotted, Figure 1. Each point represents a simple count of the number of correct responses at each concentration of taste substances.

It is evident that all of the taste substances were less easily detected in oil than in water solution. This was true even in the case of quinine which is more soluble in organic media than in water. Taste substances in water plus cellulose were less readily detected than in water alone, but more easily

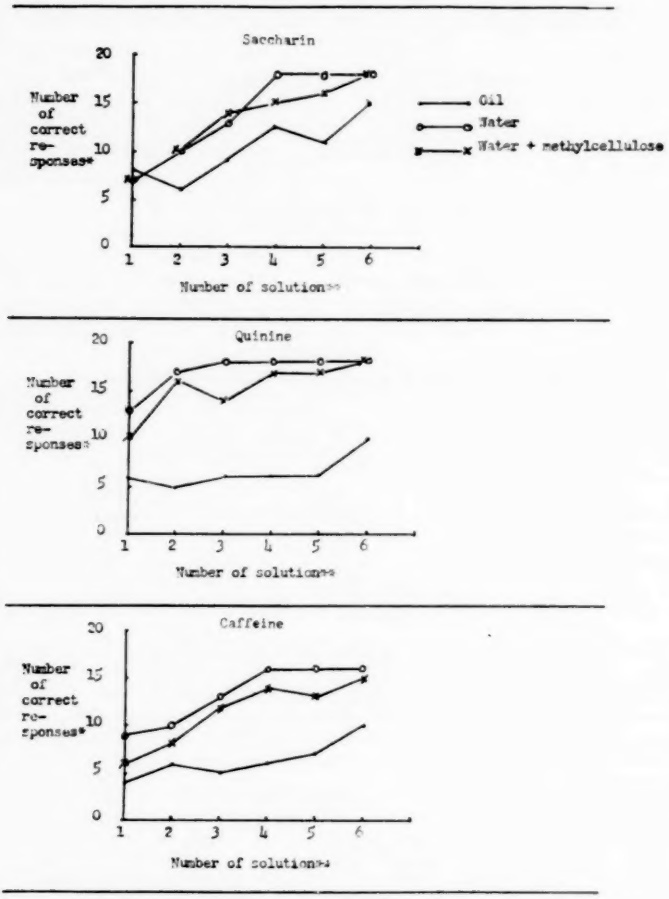

Figure 1. Perception of taste of bitter and sweet substances in oil, water and water plus methylcellulose media.

* 18 possible.
** See Table 2 for concentration.

than in oil, although the viscosity of the cellulose-water and of the oil was the same.

The taste substances in lipid solution penetrated less readily to the taste receptors than the same substances in water solution. Apparently it was necessary for the substance to dissolve in the saliva before it could be tasted. The final "yes" or "no" answers at each concentration, were arrived at only after considerable tasting and retasting, — especially in the case of the oil solutions. When in oil, the bitter substances were especially hard to detect.

Interpreting these results in the light of the research reported in the literature, it appears that water solubility is of first importance, even though accompanying lipoid solubility is a contributing factor to taste perception, as pointed out by Moncrieff *(4)*. It appears logical to conclude, furthermore, that the bitter taste, when noticeable in foods, is carried in water solution rather than in the lipids that may be present.

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ENZYME ACTION AND OFF-FLAVOR IN FROZEN PEAS
II. THE USE OF ENZYMES PREPARED FROM GARDEN PEAS[a]

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Development of off-flavor in raw and underblanched vegetables during frozen storage seems to occur as a result of enzyme action *(1)*. Use of model systems has shown that off-flavors can be produced during frozen storage by adding commercial enzymes employing macerates of enzymatically inert blanched peas *(14)*. Other work of this laboratory *(7, 8)* showed that changes which take place during the frozen storage of unblanched peas are progressive in character.

Soybean lipoxidase caused deterioration of lipids and lowered flavor scores as determined by chemical analysis and taste panel evaluation, following prolonged storage at — 17.8° C. Pancreatic lipase brought about chemical changes but off-flavors could not be determined with certainty because of flavors imparted to the pea slurry by the added enzyme itself. Addition of liver catalase and horseradish peroxidase to blanched peas produced only slight changes in quality during frozen storage.

Quality changes produced by these enzymes were of considerable interest, but it was thought that effects of enzymes from sources other than peas might not necessarily be the same as those produced by native pea enzymes.

This paper deals with the preparations of partially purified enzymes from peas and addition of these enzyme preparations to blanched pea slurries in model systems in order that their effects on quality might be studied following prolonged frozen storage.

MATERIALS AND METHODS

Perfected Freezer peas, grown on Experiment Station plots were harvested, processed, and stored at — 17.8° C. *(6)*. Blanched peas were thawed, blended with water, and mixed with enzyme preparations obtained from raw peas as described earlier (14). The ratio of blanched peas to water in the resultant slurry was 2:1. Two series of samples were prepared, one for taste panel evaluation, the other for chemical analysis. Samples of blanched pea slurry without added enzymes were included as controls in each series. A special lipase control consisting of pea catalase, pea lipoxidase, and pea peroxidase was prepared because it was impossible to separate these enzymes from the pea lipase by the procedures employed. Enzyme activity of the samples was checked prior to storage, and again after frozen storage when the residual enzyme activity was determined. Samples were stored at — 17.8 C. in cellophane bags inside waxed cartons.

Lipoxidase was prepared as follows: Lyophilized raw Perfected Freezer peas were ground to 40 mesh, mixed with 9 times their weight of cold distilled water, stirred for one hour on an ice bath, and centrifuged one-half hour at 17,000 x g. The supernatant liquid was decanted and dialyzed overnight against cold running tap water (6° C.). The dialyzed liquid was taken to 30% saturation at 4° C. with solid ammonium sulfate, allowed to stand one hour, and centrifuged as before. The supernatant liquid was then taken to 60% saturation at 4° C. with ammonium sulfate, allowed to stand one hour, and centrifuged as before. The precipitate was worked up with a small volume of cold distilled water and

[a] Approved by the Director of the New York State Agricultural Experiment Station for publication as Journal Paper No. 1118.

dialyzed overnight against cold tap water. The dialyzed material was heated rapidly to 62° C., held at 62° C. for 5 minutes, quickly cooled to 6° C. on an ice bath, and centrifuged as above. The supernatant liquid was shell-frozen in a dry ice-methyl cellosolve bath and lyophilized. Lipoxidase activity was measured at all stages of the purification procedure by manometric assay *(11, 13)*. From 100 g. of lyophilized raw peas, 1.17 g. of lyophilized lipoxidase preparation was obtained having a $Q_{O_2}{}^{10}$ of 117.5. This represented a 35-fold increase in lipoxidase activity over that of lyophilized raw peas, and a recovery of 49% of the total activity originally present in the starting material. The pea lipoxidase preparation was added at the level of 0.60 g. of lyophilized powder to 100 g. of blanched pea slurry. A one-gram sample of the resultant mixture caused the uptake of 346 μl. O_2 in 30 minutes in the manometric assay ($Q_{O_2}{}^{10} = 7.7$).

Peroxidase was prepared from lyophilized raw Perfected Freezer peas according to the method of Kenten and Mann *(3)*. Lyophilized peas were rehydrated by vacuum infiltration with 7 times their weight of cold distilled water, blended for 2 minutes in a Waring blender and the aqueous portion expressed by squeezing through several layers of cheesecloth. The crude extract was then treated in the cold with ethanol-chloroform, ammonium sulfate, and dialyzed according to Kenten and Mann's procedure. Some inert protein was removed by rapidly heating the dialyzed preparation to 59° C., maintaining this temperature for 5 min., and quickly cooling on an ice bath to 17° C., followed by centrifugation for one-half hour at 17,000 x g. The supernatant liquid thus obtained had a purpurogallin number (P.N.) of 7.0, which represented a 30-fold enrichment of peroxidase activity and a recovery of 37% of the total peroxidase activity initially present in the crude press juice. When 29.4 ml. of pea peroxidase preparation was added to blanched peas and water to make 100 g. of slurry, the resultant mixture had a P.N. of 0.138. Peroxidase activity of all fractions in the purification scheme and of the final mixture was determined by the colorimetric method of Keilin and Hartree *(2)*.

Lipase and catalase were prepared from lyophilized raw Thomas Laxton peas by methods described elsewhere *(5, 16)*. The yield of pea lipase preparation was 7.25 g. of lyophilized material from 100 g. of lyophilized peas. This preparation had a Q_{CO_2} of 14.6, which represented a five-fold concentration of lipase activity over that of the lyophilized raw peas and a recovery of 41% of the total lipase activity of the starting material. Lipase was determined manometrically using tributyrin as substrate *(9, 14)*. Pea lipase preparation was added to blanched pea slurry at the level of 6.75 g. per 100 g. of slurry. A one-gram sample of the resultant mixture caused the liberation of 261 μl. CO_2 in 30 minutes in the manometric assay ($Q_{CO_2} = 2.3$).

Pea catalase was enriched to Kat. f. 420. It was added at the rate of 13.5 ml. in 100 g. of blanched pea slurry. A 0.1 g. sample of the resultant mixture produced 2.2 ml. O_2 in the Thompson test *(12)*.

Determinations of acid numbers, peroxide numbers, and chlorophyll were made as reported earlier *(4, 6, 15)*.

Organoleptic tests were carried out with a qualified panel of 7 members. Scores were based on a 1 — 10 scale. Ten was the highest value.

RESULTS AND DISCUSSION

Analytical results of this study are summarized in Table 1. Samples of blanched peas to which native pea enzymes had been added were subjected to taste panel evaluation for flavor and color after 18 months of storage at — 17.8° C. In each case evaluation was based on comparisons with the flavor and color of blanched peas with no added enzymes, which had been stored under similar conditions. As in the previous study *(14)* in which commercially obtained enzymes were employed, addition of enzymes to blanched peas produced off-flavors ranging in intensity from *mild* to *extremely objectionable*. Mild off-flavor was produced by added peroxidase. The more objectionable flavor changes were found in samples to which lipase, lipoxidase, and catalase had been added. The flavor produced in the added catalase was the most objectionable. A mixture containing lipoxidase, catalase, and peroxidase in the amounts present in the pea lipase preparation also produced an objectionable off-flavor when added to the blanched peas.

TABLE 1

The effect of added pea enzymes on quality of bleached peas

Treatment of sample	Acid No.	Peroxide No.	Residual enzyme activity				Percentage of original activity				Chlorophyll % destroyed	Taste panel	
			Lipase $\mu l.\ CO_2$	Lipoxidase $\mu l.\ O_2$	Catalase ml. O_2	Peroxidase P.N.	Lipase	Lipoxidase	Catalase	Peroxidase		Flavor	Color
Lipase	14.3	4.6	129	56	3.5	0.08	49	32	159	57	2.5	3.4	4.6
Lipase blank	4.4	1.1	2	—	—	—	1	—	—	—		7.1	8.4
Special lipase control	7.9	5.2	32	65	1.1	0.06	12	38	50	43		3.4	5.6
Lipoxidase	7.4	5.1	0	224	0	0.05	0	130	0	36	27.5	4.4	5.6
Lipoxidase blank	5.4	1.8	—	0	—	—	—	0	—	—		7.0	7.9
Special lipoxidase control	5.3	2.0	—	0	—	0.03	—	0	—	21		5.5	6.3
Catalase	10.8	4.9	0	0	2.0	0.01	0	0	91	7	10.6	1.9	5.3
Catalase blank	6.3	2.0	—	—	—	—	—	—	0	—		7.1	7.9
Peroxidase	6.8	1.1	0	4	0.05	0.13	0	2	2	93	5.2	6.0	6.7
Peroxidase blank	4.0	0.9	—	—	—	0.01	—	—	—	7		7.9	8.1
Original activity prior to frozen storage			261	173	2.2	0.14	100	100	100	100			

Changes in color brought about by the action of added pea enzymes were readily detected by the taste panel, and also evidenced by losses of chlorophyll measured spectrophotometrically. The greatest amount of chlorophyll destruction, 27.5%, was observed in the lipoxidase-treated sample.

Deterioration of lipids was evidenced by increases in titratable acidity and presence of organic peroxides in the ether extracts of lyophilized samples. Increases in acid numbers were quite meager when compared with the very large acid numbers caused by the addition of pancreatic lipase in the earlier experiments with commercial enzymes *(14)*. Catalase-treated blanched peas showed an increase in acid number, as did the lipase-treated peas. Peroxide numbers were of the same general order of magnitude as had been obtained with the commercial enzymes *(14)*. Positive peroxide numbers were obtained by treatment with all four pea enzymes, the lower values from samples treated with peroxidase and catalase, and higher values from samples treated with lipoxidase and lipase. It must be remembered that the lipase used was contaminated with lipoxidase.

Analyses of samples for residual enzyme activity following 18 months of frozen storage indicated that three of the enzymes, lipoxidase, catalase, and peroxidase, had maintained nearly full activity throughout the storage period. The lipase-treated sample contained less than one-half of its original lipase activity prior to storage.

Off-flavors produced in this study were generally similar to those occurring during the frozen storage of raw peas *(6)*. All samples containing added enzymes were tasted prior to freezing. The enzyme preparation, *per se*, contributed nothing to the taste of the final products.

The color change brought about by lipoxidase action through conversion of chlorophyll to colorless products *(14)* was again demonstrable when lipoxidase-containing samples were mixed with linoleic acid during determination of residual lipoxidase activity. The pea tissue fragments of such samples showed a marked degree of bleaching when compared with blanched pea control samples.

The enzymatic purity of the various pea enzymes used in this study was ascertained. Pea lipoxidase was found to contain no lipase or catalase activity. The lipase preparation, on the other hand, was found to be a mixture of all 4 enzymes, possessing strong lipoxidase and catalase activity. The heat treatment step employed in preparing pea lipoxidase was found to cause nearly complete inactivation of pea lipase *(16)*. The activity of pea lipase was found to be greatly diminished by such agents as ammonium sulfate and acetone *(7)*. Inasmuch as the purification of the other 3 enzymes involved salt fractionation with ammonium sulfate, and in addition the use of a chloroform-ethanol mixture in preparation of peroxidase, it is logical to assume that only negligible amounts of lipase activity would have remained in the purified preparations of pea lipoxidase, catalase and peroxidase. Analyses of the various samples for residual lipase activity after prolonged frozen storage bore this out. Samples treated with the other 3 enzymes were found to be devoid of lipase activity, with the exception of the special lipase control sample which contained a mixture of lipoxidase, peroxidase, and catalase. Peroxidase activity following 18 months of frozen storage was present in all of the enzyme-treated samples and to a small extent in the blanched pea con-

trol. Regeneration of peroxidase is not uncommon in vegetables held in frozen storage *(14)*.

Of the 4 enzymes used, only pea lipase suffered any appreciable loss of activity during frozen storage, amounting to 52%. It was impossible to determine when this deterioration of lipase had occurred, but the fact that very little increase in acid number was observed in lipase-treated samples may be best explained by the lack of extensive lipase action during storage. In contrast the pancreatic lipase used in the previous study *(14)* retained 96% of its original activity during 17 months of frozen storage and produced very large increases in titratable acidity.

The finding of positive peroxide numbers, substantial losses of chlorophyll, and lowered flavor scores in lipoxidase-treated pea samples was taken as further substantiation of the role played by lopoxidase in production of off-flavors and other deteriorative quality changes during frozen storage *(10, 13, 14)*.

The role of lipase is more difficult to assess, primarily because of the relative enzymatic impurity of the pea lipase preparation. Whereas the lipase-treated samples did show a moderate increase in acid number, the catalase-treated sample showed nearly as great an increase. Furthermore, as mentioned, the pea lipase preparation was a fairly good source of peroxidase and a good source of lipoxidase and catalase, both before and after frozen storage.

Treatment of blanched pea slurry with pea lipase must therefore be considered as the addition of a mixture of enzymes, the effect of which was to produce disagreeable off-flavors in the product after 18 months of storage at — 17.8° C. The same was true of the special lipase control sample which was found to possess activity of all 4 enzymes after storage, and which also was given a low flavor score by the taste panel.

The action of pea catalase in producing off-flavor when added to blanched peas was certainly much more profound than the corresponding action of liver catalase had been in the previous study *(14)*. The sample containing added pea catalase preparation possessed the most disagreeable off-flavors of any samples in this present study. Furthermore, this catalase preparation also resulted in increased acid number and peroxide number in the ether extracted lipids, following frozen storage. The catalase-treated sample was found to be devoid of any residual lipoxidase or lipase activity and to contain only a small amount of residual peroxidase activity after 18 months' frozen storage. Presence of organic peroxides may have been due to non-enzymatic catalysis *(14)* but the production of acid which also has been observed when blanched peas were treated with liver catalase, remains unexplained.

In this connection, however, it might be well to consider the work of Keilen and Hartree *(3)*. These authors noted the formation of aldehyde and acid from a system containing xanthine oxidase, alcohol, and catalase. It is within the realm of possibility that a system similar to this could be responsible for the formation of the acid in the slurries containing added pea catalase.

These studies, in which both commercially available enzymes and pea enzymes have been added to blanched pea slurry in model systems, have involved the 4 enzymes which have been thought to be associated with quality changes during frozen storage. The four are lipoxidase, lipase, catalase, and peroxidase. Lowered flavor scores were obtained with all of these enzymes. The

greatest changes in flavor score were associated with the addition of soybean lipoxidase as far as the commercial enzymes used were concerned, and with preparations of lipoxidase, lipase, and catalase prepared from raw peas. In the latter the catalase prepared from raw peas produced the most offensive off-flavor. The results of enzymic action have been demonstrated in chemical analyses for increases in titratable acidity, development of organic peroxides, and destruction of chlorophyll, all of which are associated with deterioration of quality in frozen peas.

Results of these studies do not, however, preclude the involvement of other enzymes, as yet unidentified. These may be present in raw peas and may retain their activity through the various fractionation procedures employed in the partial purification of the 4 above-named enzymes. It seems likely that off-flavor production in frozen raw vegetables is quite complex in nature and that the concerted action of several or many enzymes is involved.

SUMMARY

Lipoxidase, lipase, catalase, and peroxidase have been obtained in partially purified form from lyophilized raw peas.

Adding these partially purified preparations of native pea enzymes to blanched peas resulted in the production of off-flavors following prolonged frozen storage. The greatest change in flavor score was associated with added catalase, but lipoxidase and lipase produced pronounced off-flavor. Peroxidase produced only a mild change in flavor which was not objectionable.

Changes in green color were brought about by adding all four enzymes. The greatest lowering of visual color scores was produced by lipoxidase and lipase.

Deterioration of lipids was evidenced by formation of peroxides and moderate increases in acid number.

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ENZYMATIC HYDROLYSIS OF NARINGIN, THE BITTER PRINCIPLE OF GRAPEFRUIT*

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Naringin, the 7-rhamnosido-β-glucoside of 4',5,7-trihydroxyflavanone, the main bitter principle of grapefruit, was first discovered in 1857 by DeVry *(3)* in the flowers of grapefruit trees growing in Java. This glycoside is also found in the fruit, chiefly in the albedo and segment membranes. It is apparently most abundant in immature fruit, the content decreasing as the fruit ripens. Juices and other grapefruit products high in naringin concentration are excessively and disagreeably bitter. A practicable method of reducing such excessive bitterness is highly desirable.

A number of different methods have been proposed for removing naringin from grapefruit products. Acid hydrolysis of the glycoside yields rhamnose, glucose and the non-bitter aglycone, naringenin, but such a procedure would be too drastic for practical application. Also, under the proper conditions of pH and temperature, activated carbon will almost completely remove naringin from solution *(1)*, but desirable flavor constituents are also removed and this method likewise not too feasible. The most suitable method would be the enzymatic hydrolysis of naringin. This method offers the advantages of mild conditions and a minimum number of required manipulations.

Hall *(5)* reported in 1938 the isolation of an enzyme from celery seeds which hydrolyzed naringin *in vitro*, at pH 7 and 37° C., to naringenin and the intact disaccharide. He also reported the occurrence of a similar, or identical enzyme in the leaves of the tree, *Citrus decumana*, and in smaller amounts in the region between the flavedo and albedo of grapefruit. He found that neither rhamnodiastase (which catalyzes the hydrolysis of rutinose(β-L-rhamnosido-D-glucose) from the pentahydroxyflavone glycoside, rutin) nor emulsin (a well known β-glucosidase) had any effect on the hydrolysis of naringin. Ting *(8)*, in repeating the work of Hall, was able to obtain only very low yields of the enzyme from leaves of grapefruit trees.

Ting *(8)* found, however, upon treating grapefruit juice with relatively high concentrations of Pectinol (a commercial pectic enzyme preparation of fungal origin) to facilitate filtering, that the total flavonoid glycoside content of the juice was greatly reduced, as determined by the reduction in intensity of the yellow color produced by the Davis alkaline-diethylene glycol reagent *(2)*. Naringin in aqueous solution was also found to be completely hydrolyzed at pH 4 by similar concentrations of Pectinol, yielding, as the only products, glucose, rhamnose, and naringenin.

The observations of Ting with Pectinol were confirmed in this laboratory, and it was deemed worthwhile to investigate the possibility of producing more active "naringinases" and to study further the properties of such an enzyme or enzymes. Therefore, a program was undertaken to screen a large number

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of microorganisms for the capacity to produce enzymes capable of catalyzing the hydrolysis of naringin, and thus potentially able to debitter products containing this material.

EXPERIMENTAL

Preparation of enzymes. The crude enzymes employed in this study were prepared from cultures of a series of microorganisms. Growth media were inoculated from slant cultures and then incubated for various times. At the end of the growing period crude culture extracts were obtained and used without further purification for the preliminary evaluations of naringinase activity.

When it was desired to isolate the enzymes to allow for more detailed study of properties, partial purification was effected by treating the culture extracts with alcohol to yield precipitates, subsequently recovered by filtration and dried in air.

In cases where it was necessary or desirable to free the "naringinase" from associated pectic enzymes, either culture extracts, or aqueous solutions of alcohol precipitates were treated with urea, according to the method of Smythe and Neubeck *(7)* for the preparation of pectinase-free diastase. The naringinase activity could thereby be retained intact, while the pectic enzymes suffered essentially complete destruction.

The emulsin used in a number of experiments was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio.

Determination of activity. Preliminary determinations of naringinase activity were made with a standard, aqueous substrate solution consisting of 0.05% recrystallized naringin, 1.5% citric acid, and sufficient sodium hydroxide to bring the pH to 4.0. To 25 ml. aliquots of this solution were added 0.2 ml. of culture extract, and the reaction mixture then maintained at 50° C. during the course of the experiment. When desired, the initial substrate concentration, enzyme concentration, buffer concentration, pH, or reaction temperature could readily be varied.

To measure the change in naringin concentration the Davis method *(2)* was used. This method is based on the intense yellow color, as determined spectrophotometrically at 420 mμ, produced by naringin upon reacting with alkaline diethylene glycol. Since the aglycone, naringenin, produces far less color, samples taken at intervals during the hydrolysis of naringin to naringenin show a continuous decrease in color on reacting with the reagent. The extent of hydrolysis to naringenin can then be determined from a standard curve, prepared using known concentrations of glycoside and aglycone.

Units of naringinase activity were calculated on the basis of the time required to give 50% hydrolysis of the standard substrate solution at 50° C. by a given concentration of enzyme.

Presence of pectic enzymes in the crude naringinase preparations was demonstrated by the reduction in viscosity of a pectin containing substrate solution at pH 4.5.

Debittering of grapefruit juice. Various samples of fresh and canned grapefruit juice were treated under various conditions of temperature and enzyme concentration, and the reaction followed both by the Davis method and by taste tests.

RESULTS AND DISCUSSION

About 100 cultures were screened for the production of "naringinase", and a number of these yielded extracts with significant activity. One of these extracts in particular contained an especially high order of activity and was selected for further study. The crude culture extract was therefore treated with urea to destroy the pectic enzymes, and an alcohol precipitate then prepared. The activity of the dried enzyme preparation was about 30 times that of the original culture extract. This enzyme preparation will henceforth be referred to as Naringinase C.

In Figure 1 there is illustrated a typical Naringinase C hydrolysis of the standard substrate solution. It can be seen that the curve is linear up to 70% hydrolysis, and the rate then decreases steadily until the reaction is essentially complete. Such apparent linearity to over 50% hydrolysis has also been observed with a number of other crude enzyme preparations.

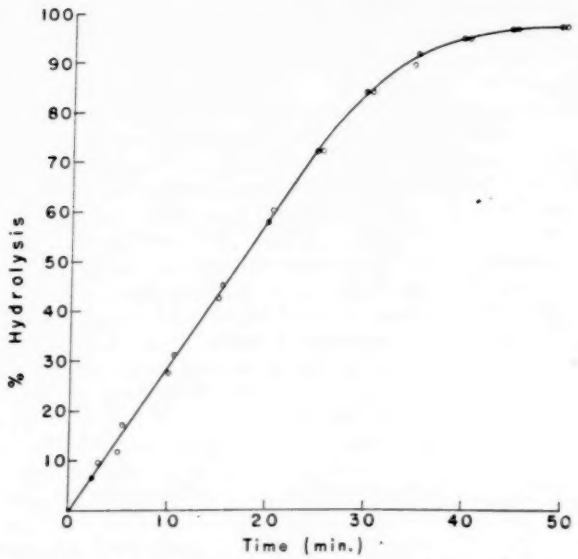

Figure 1. Hydrolysis of the standard substrate solution by Naringinase C.

Partly on the basis of Ting's *(8)* findings with Pectinol, a pH of 4.0 was selected for that of the standard substrate solution. However, as the pH optimum often varies for the same enzyme activity from different sources, it was of importance to determine that of Naringinase C. The activity was thus determined over a fairly wide range of hydrogen ion concentration and at temperatures of 30° C. and 50° C. It can be seen from Figure 2 that the optimum for Naringinase C at the higher temperature is near 4.0, while at the lower temperature it is apparently close to 4.5. However, in both cases the activity is fairly close to the maximum over the pH range of 3.5-5.0, and this enzyme preparation, therefore, should be highly active in grapefruit juices where the pH is not normally too far removed from 3.5.

Although in the preliminary screening tests the crude culture extracts were always tested at 50° C., they were sometimes also tested at 30° C. In all cases the activity was considerably higher at 50° C., indicating good heat stability for naringinase activity at this relatively high temperature. In Figure 3 are given the activities for Naringinase C over the temperature range of 21.5-70° C., and for a reaction time of 30 minutes. The actual maximum cannot be determined with the limited amount of data available, but it is probably somewhere between 55° C. and 60° C. for reactions of short duration. At 70° C. the activity was calculated on the basis of the initial reaction rate, but the value does not have too much significance as the reaction ceased after only 20% hydrolysis. At 60° C. the reaction went essentially to completion. Between 20° C. and 30° C. the initial reaction rate increased almost twofold, a Q_{10} not uncommon for enzymatic reactions. As the temperature was raised, however, the Q_{10} decreased significantly, presumably due to denaturation of the active protein, and between 50° C. and 60° C. it was only about 1.2.

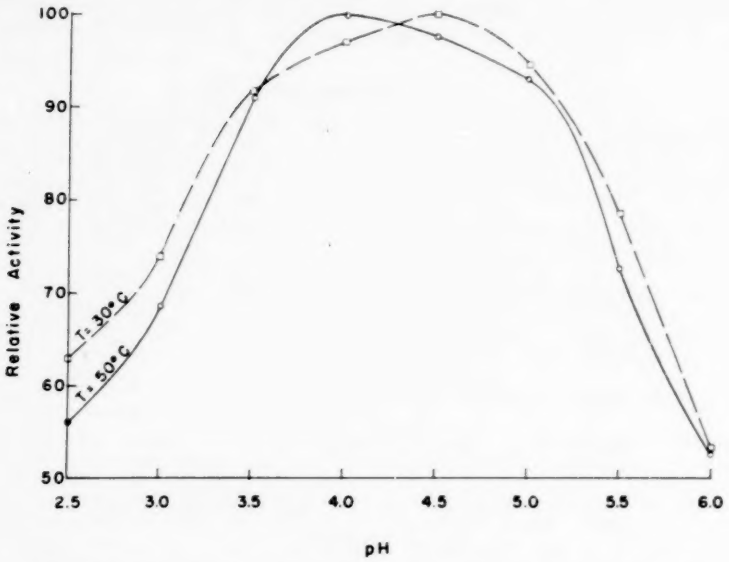

Figure 2. Effect of pH on Naringinase C activity at 30° C. and 50° C.

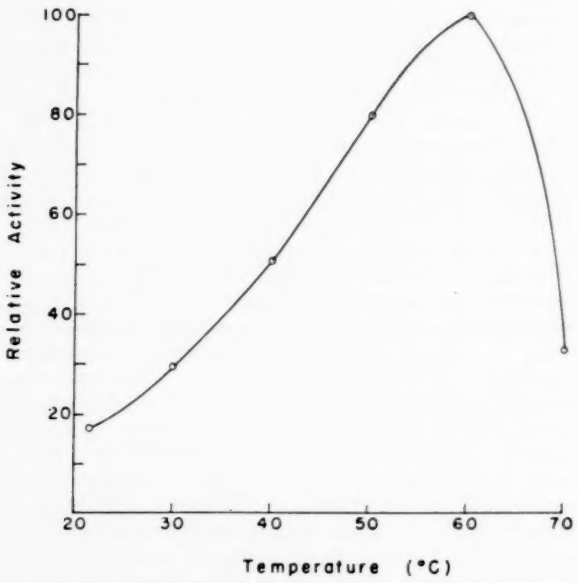

Figure 3. Effect of temperature on Naringinase C activity.

Crude culture extracts of the organism used to produce Naringinase C, and which had a pH of 5-6, were possibly even more stable and could be heated at 60° C. for 4 hours with only 16% loss in activity. At 65° C., however, 83% of the activity was lost after 1 hour. Neither the culture extracts, stored at 5° C., nor Naringinase C, stored at room temperature, have shown any loss in activity over a year's period of time.

Although Naringinase C apparently had the requisite properties for the rapid hydrolysis of naringin at low enzyme concentrations, it was found that on treating grapefruit juice with this preparation, the Davis test indicated rates of hydrolysis of from only 10-30% of those obtained with equal concentrations of naringin in aqueous solution. It was conceivable that these low rates were due to the naringin not being readily available for enzymatic hydrolysis in the juice. This possibility was eliminated, however, when it was found that purified naringin, added to juice previously clarified and treated with excess enzyme to remove essentially all of the original naringin, was still hydrolyzed at rates of the same low order of magnitude.

Although Ting *(8)* found that the naringinase activity of Pectinol, a preparation of a different origin and with unlike enzymatic properties, was only very slightly inhibited by the concentrations of glucose found by him to be present in grapefruit juice, it was nevertheless considered likely that this, or some other sugar might be responsible for the apparent low orders of activity given by Naringinase C in grapefruit juice. Therefore, a series of sugars were added to the standard substrate solution at concentrations of 2% and the reaction rates redetermined. Although sucrose, fructose and other sugars were only slightly, if at all inhibitory, glucose decreased the reaction rate by over 60% at this concentration, and caused still further decreases at higher concentrations, as can be seen by inspection of Figure 4. Thus, the concentrations of glucose likely to be present in grapefruit juices can adequately account for the low activity of Naringinase C, as determined by the Davis method.

Although the Davis method indicated low rates of hydrolysis of naringin in grapefruit juice, it was discovered that the juice was nevertheless rapidly debittered by Naringinase C, bitterness no longer being detectable even though the extent of the reaction was sometimes apparently only 20% or less. Thus, it was evident that there was a definite lack of correlation between the rate of debittering of grapefruit juice and the Davis test, and this could have been due to any of several factors. Griffith *(4)*, who was working with naringinase preparations supplied by the Rohm and Haas Company, suggested that enzymatic hydrolysis might change the reaction of other flavonoids in grapefruit juice to the Davis reagent, thereby causing interference with the specificity of the test. However, it was also possible that the conversion of naringin to naringenin, the reaction measured by the Davis method, was not the precise reaction involved in the debittering of the grapefruit juice.

At about this same time it was found that although high concentrations of emulsin neither catalyzed the hydrolysis of standard naringin solutions when present as the only enzyme, nor increased the rate of hydrolysis when added in conjunction with Naringinase C, it did, however, reverse the glucose inhibition of Naringinase C. Since Nystrom, Williams and Wender *(6)* had shown that emulsin was able to catalyze the hydrolysis of naringenin-7-β-glucoside (prunin), it was soon suspected that in the presence of inhibitory

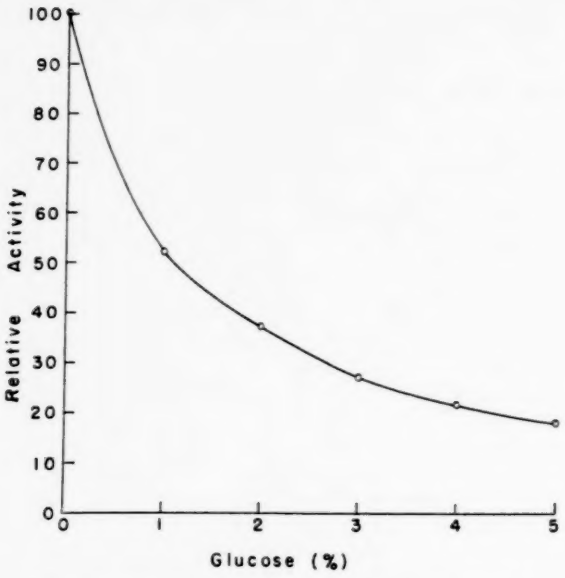

Figure 4. Effect of glucose concentration on Naringinase C activity.

concentrations of glucose this glucoside accumulated as an intermediate in the enzymatic hydrolysis of naringin to naringenin.

To verify the possible accumulation of prunin in enzymatic hydrolyses carried out in the presence of glucose, the following experiment was carried out: To the standard substrate solution was added sufficient glucose to give a concentration of 5%, the temperature brought to 50° C. and Naringinase C then added. After 30, 45 and 60 minutes aliquots were withdrawn and rapidly heated to 100° C. to destroy the enzyme. A duplicate reaction was carried out simultaneously, but without the added glucose. The Davis method was used to calculate the apparent degree of hydrolysis at each time interval, and these values are given in the middle two columns of Table 1. It can readily be seen that only slight hydrolysis in the presence of glucose was indicated, while extensive hydrolysis occurred when no sugar was added.

Each of the aliquots from the reaction mixture containing glucose was then adjusted to pH 5 and an excess of emulsin added. The solutions were maintained at 35° C. until no further hydrolysis was indicated. In the last column of Table 1 are given the values for the total extent of hydrolysis, again as determined by the Davis method, for each of the emulsin treated aliquots. It is immediately evident that the total amount of hydrolysis in each of the aliquots after the emulsin treatment was now essentially the same as that when no sugar was added.

From the above, and previous experiments, it can be concluded that Naringinase C contains at least two glycosidases. One, a rhamnosidase, is specific for the hydrolysis of the bond between rhamnose and glucose in

TABLE 1
Evidence for accumulation of prunin in Naringinase C hydrolysis partially inhibited by glucose.

Time of naringinase C hydrolysis	% Hydrolysis		
	5% Glucose	No glucose	After emulsin treatment
0′	0	0	0
30′	5	38.5	40
45′	10.5	63	56.5
60′	17	76.5	69.5

naringin and yields prunin and rhamnose, and the other, a β-glucosidase, is specific, as is emulsin, for the hydrolysis of the bond between the glucose and the aglucone in prunin and yields naringenin and glucose. Also, since emulsin does not increase the overall rate of hydrolysis of naringin to naringenin by Naringinase C in the absence of glucose, it is evident that in this case, the first step, the conversion of naringin to prunin, is rate determining and the intermediate does not accumulate. However, in the presence of inhibitory concentrations of glucose, the second step, the conversion of prunin to naringenin, also becomes rate determining and prunin accumulates. Under these latter conditions the addition of emulsin will then increase the rate of the overall hydrolysis by aiding in the removal of the intermediate.

Since, in the presence of inhibitory concentrations of glucose, the Davis method indicates very low rates of hydrolysis even though the rate of the first step is essentially the same as in the absence of glucose, it is obvious that this method cannot be used to measure the rate of formation of prunin, but only the rate of formation of naringenin. As stated previously, these two rates will be identical in hydrolyses catalyzed by Naringinase C in the absence of inhibitors of the β-glucosidase component, since it is the first step that is entirely rate determining under these particular conditions.

In one further experiment, a 0.1% solution of naringin was treated with Naringinase C in the presence of 0.25% δ-gluconolactone, a material found to be over 20 times more potent than glucose as an inhibitor. After the time estimated to be necessary for the complete removal of the rhamnose moiety from the naringin, the reaction was stopped by holding the solution near 100° C. for several minutes. The Davis test indicated only a small amount of hydrolysis. The solution was then placed in the refrigerator for several days, and at the end of this time the amorphous precipitate that had formed was recovered. This material was not bitter, but unlike naringenin, gave a significant color with the Davis reagent, and was very rapidly hydrolyzed by both emulsin and Naringinase C.

The above results lend further support to the conclusion that debittering is a function of the conversion of naringin to prunin, and that the further hydrolysis to naringenin is not required. Since this conversion to prunin is not significantly inhibited by the sugar in grapefruit juice, the juice can be rapidly and successfully debittered by Naringinase C. Although the Davis method cannot be used to determine directly the rate of debittering by Naringinase C, since it only measures the overall conversion of naringin to naringenin, it can be used to determine the rhamnosidase activity in the standard substrate solution. As illustrated previously, with this particular

enzyme system under the stated conditions, the rhamnosidase activity is rate determining. Therefore, it should be possible to predict quite accurately the rate of debittering of grapefruit juice by Naringinase C with this prior knowledge of the rhamnosidase activity, as determined on isolated naringin.

SUMMARY

A large number of microorganisms were screened for the capability of producing enzymes able to hydrolyze the bitter glycoside, naringin. A number of the organisms produced such enzymes, and one especially active culture was selected for detailed study.

A partially purified enzyme preparation, Naringinase C, was found to rapidly hydrolyze naringin *in vitro* in the pH range 3.5 to 5.0 and at temperatures of from 20-50° C. This preparation also rapidly debittered natural grapefruit juices.

It has been concluded that only the partial hydrolysis of naringin is required for debittering, the removal of the rhamnose moiety to yield prunin (naringenin-7-β-glucoside) being sufficient. The Davis method, used in the past to measure the rate of hydrolysis of naringin, is not suitable for the direct measurement of debittering since it only measures the overall hydrolysis to the aglycone, naringenin, and in the presence of the glucose in grapefruit juice the enzymatic hydrolysis of prunin to yield naringenin is comparatively slow.

Acknowledgment

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USE OF COBALT GLASS DOSIMETRY IN ELECTRON BEAM RADIATION OF FOODS[a, b]

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(Manuscript received May 31, 1958)

One problem in the experimental radiation of foods is the difficulty of obtaining an accurate estimation of the dose actually received. This is especially true of electron beam radiation because of limited penetrating power and consequent non-uniform dose distribution. The Fricke dosimeter *(3)*, essentially a tube of ferrous sulfate solution, has proven to be a very satisfactory working standard for gamma radiation, but is too bulky for similar use with electron beams where thin layers of food are to be treated.

In the studies to be described, tiny cobalt glass dosimeters were inserted in samples of a food product, prebaked pancakes. These were radiated by electrons at various dose levels and the respective increases in spectrophotometric absorption coefficient determined. Similar food-dosimeter combinations were radiated at various dose levels in a gamma source which had been accurately calibrated by ferrous dosimetry. Increase in absorption coefficient was plotted versus reported dose for each source in order to provide reference curves for future experiments.

MATERIALS AND METHODS

Cobalt dosimeter glasses. These were purchased from Bausch and Lomb Optical Co. and measured 6 mm x 15 mm x 1.5 mm. The exact thickness of each glass was determined with a micrometer caliper, and its optical density was read at 450 and 500 mμ in a Bausch and Lomb Spectronic 20 colorimeter using a special dosimeter holder.

Pancakes. These were prepared by a commercial formula, with a dosimeter glass placed in the center of each. They were then heat-sealed in individual transparent envelopes. The film used consisted of an extrusion of polyethylene on Mylar. Those intended for electron radiation were left in this form; those intended for gamma radiation were further sealed in No. 2 cans, one envelope to a can. Preparation and packaging took place 2 or 3 days before radiation, the packaged products being kept frozen during the intervening period.

Radiation. Radiation at 6 dose levels ranging from 0.465 to 3.72 x 10^6 rads was requested at the General Electric Company 2 Mev resonant transformer facility in Milwaukee. Two samples were radiated at each level. A specific gravity of 0.6 and total thickness of 0.5 inches were used in calculating the exposure needed for the dose requested. Radiation at the same 6 dose levels was requested at the Argonne National Laboratory gamma facility, which uses spent fuel-rods and ferrous dosimetry *(4)*.

Determination of absorption coefficient. Optical densities of the exposed glasses were read at 450 and 500 mμ in the colorimeter 3 to 4 hours after radiation. The increase in absorption coefficient due to radiation was calculated for each glass and plotted versus reported dose on logarithmic co-ordinate paper as described by Kreidl and Blair *(1, 2)*. Since electron beam dosage was reported at the food surface, these data were recalculated

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[a] This paper reports research undertaken in cooperation with the Quartermaster Food and Container Institute for the Armed Forces, QM Research and Engineering Command, U. S. Army, and has been assigned number 887 in the series of papers approved for publication.

[b] Presented at the Eighteenth Annual Meeting, Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

to show the average dose to each dosimeter using the curve of intensity versus penetration in Figure 1, which was drawn from data furnished by General Electric Company. In this figure, beam intensity is plotted for various depths of penetration in a material of unit specific gravity. It is thus possible to determine intensity at any given depth by laying off the appropriate intercept along the horizontal axis. For materials of other than unit specific gravity, the equivalent intercept is calculated by multiplying the actual depth of material by its specific gravity. In laying off the intercepts shown, the following assumptions were made: Specific gravity of pancake crumb 0.6, specific gravity of cobalt glass 2.5, depth of pancake crumb above and below glass 0.25 inches, and depth of glass 1.5 mm. Entrance and exit doses to the glass thus appear as 91% and 18%, respectively, of the reported dose to the pancake surface. Since the curve is practically linear in this area, the average dose to the glass was taken as the mean of these two values or 54.5%

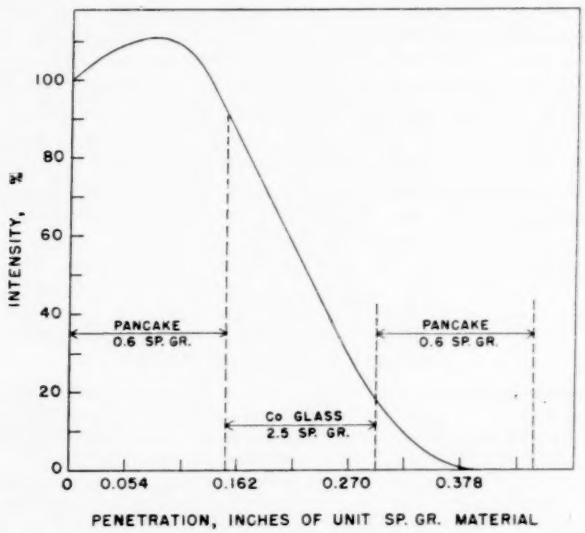

Figure 1. Relationship of beam intensity to depth of penetration in unit specific gravity material at General Electric Co. 2 million-electron-volt resonant transformer facility.

RESULTS

Thickness of the dosimeters showed little variation from glass to glass and averaged 1.506 mm. Optical density before radiation also showed little variation and averaged 0.243 at 450mμ and 0.611 at 500 mμ. Absorption coefficients for zero radiation were thus 3.71 and 9.33, respectively.

Increase in absorption coefficient at 450mμ and at 500mμ are plotted versus reported dose in Figures 2 and 3, respectively, for both sources, the solid line for the electron source in each case representing reported dose at the food surface and the broken line representing the average dose to the cobalt glass calculated as described earlier. The 6 reported dose levels are not identical for the two sources as was originally requested. An error in calculating exposure to the electron beam was discovered and reported several days after the irradiations had been completed, and electron dosages were corrected by multiplying each by a factor of 1.38.

DISCUSSION

It will be noted th . a reasonably linear relationship exists between dose and change in absorption coefficient in the range of 1 to 4 megarads, and

that the slope is the same for both sources. Reported dose of electrons appears to be about double that of gamma rays for the same increase in absorption coefficient. While the electron dose is still higher than the gamma dose after correction for loss of intensity upon penetration, the difference is reduced from 110% to 13%, thus approaching the range of error involved in the method itself as used here.

The use of glass dosimeters in representative samples would appear to provide one convenient and practical means of obtaining an approximate indication of dose received. In practice it might be necessary to run a working calibration curve for each product similar to those represented by the solid lines in Figures 2 and 3, in order to check the validity of the assumptions involved in calculating average dose.

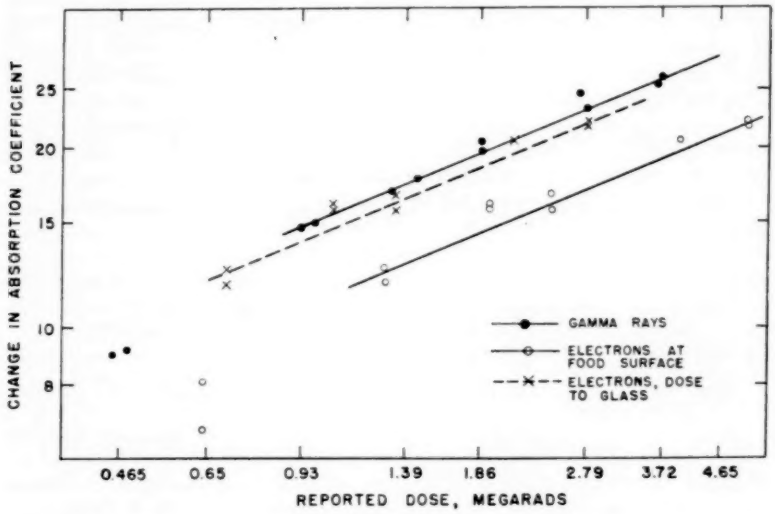

Figure 2. Effect of variation in dosage upon the absorption coefficient of cobalt glass at 450 mμ.

It should be pointed out that the scope of this study was restricted to the practical use of glass dosimeters placed in samples of food as a means of checking dose received. It was assumed that equal absorption coefficients from the two types of radiation meant equal dose to the glass, and further that the gamma doses had been correctly determined by ferrous dosimetry.

SUMMARY

Prebaked pancakes containing cobalt glass dosimeters were radiated at various dose levels by gamma rays from an accurately calibrated spent fuel rod source and by electrons from a resonant transformer. Standard curves were prepared by plotting on logarithmic paper the change in absorption coefficient at 450 mμ versus reported dose. On this basis each type of radiation showed a linear dose-absorption relationship in the 1-4 megarad range.

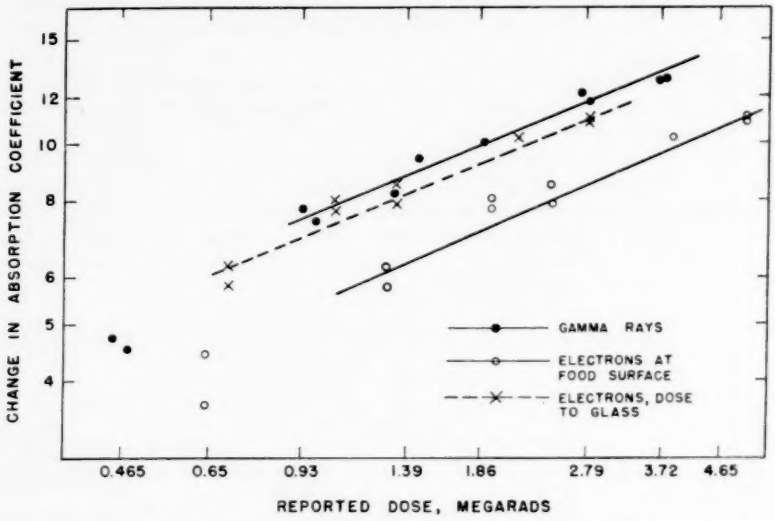

Figure 3. Effect of variation in dosage upon the absorption coefficient of cobalt glass at 500 mμ.

Correction of the electron curve for beam attenuation in the dosimeter glass brought it into virtual agreement with the gamma ray curve.

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ACCEPTABILITY OF FRESH AND PRECOOKED RADIATED MEATS[a, b, c]

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(Manuscript received May 31, 1958)

Subjecting foods to ionizing radiations has been accepted *(2, 5, 8)* as an effective means of destroying microorganisms and thereby effecting preservation. The importance of the microbiological aspects *(5)* is paramount, but the radiated product must also be acceptable and nutritionally adequate. It must be acceptable not only after radiation but also after a period of storage *(5)*. Since the storage temperatures in military supply lines may vary, the products must not be adversely affected by extremes in temperature. It is curious but true, however, that during storage an improvement in the flavor of certain foods has been noted while in others, the flavor and acceptability have been lowered.

Another facet of the problem relates to the enzymes. It has been reported *(2, 5)* that the inactivation of enzymes by radiation requires the application of considerably greater dosages than does the inactivation of spore-forming bacteria. In view of this, and the fact that increasing levels of radiation result in decreasing acceptability of the products *(9)*, it would appear that the inactivation of the enzymes in certain foods must be accomplished by means other than radiation alone.

The present work was initiated to determine the effects of storage on the flavor and acceptability of fresh and precooked radiated meats. In the former, the enzymatic systems would presumably be operative and in the latter, they would be inactivated. Certain changes in the protein complex of the meat was also determined in order to clarify the situation in respect to the enzymic stability of the meat during the storage period.

EXPERIMENTAL

Fresh beef chuck roasts which were radiated without further treatment were obtained from U. S. Good carcasses, aged 7 days. Pork shoulder roasts were cut from fresh carcasses. Beef steaks and pork chops used were cut from the respective carcasses as indicated above. The bacon was a commercially available "thick-slice" of normal commercial cure, purchased directly from the processing plant.

Meats which were precooked before radiation were obtained from a commercial meat packer in Ogden, Utah. Beef roasts and steaks were obtained from U. S. Prime carcasses, aged 7 days. Beef roasts were boned, rolled, and tied prime ribs; the steaks, New York cuts. Pork roasts were boned, rolled, and tied fresh pork legs. Pork roasts and chops were obtained from fresh carcasses.

The meats which were not precooked were placed in No. 10 plain tin cans, then sealed and frozen at −20° F. No attempt was made to exclude air. The cans of meat were

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[b] Presented before the Eighteenth Annual meeting of the Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

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shipped, with dry ice, to the Materials Testing Reactor, Arco, Idaho and exposed to the gamma grid for times sufficient to accumulate 1.9 and 2.8 megarad. Suitable controls which were not radiated were similarly shipped to the MTR. Return shipment was with dry ice and upon receipt, the control samples were placed at 0° F. and the irradiated samples at 72° F.

The meats to be precooked were shipped at 36° F. to 34° F. storage rooms at the Dugway Proving Ground, Utah. Arrangements were made to utilize Army field ranges for precooking prior to radiation at the Radiation Laboratory, Dugway Proving Grounds.

Beef steaks and beef roasts were precooked to an internal temperature of 160° and 180° F. as indicated by thermocouples. Steaks were fried on the grills on top of the Army field ranges and the ribs roasted in an oven at 350° F. in uncovered pans. The steaks required 20 and 25 minutes and the beef roasts 3 and 3½ hours to reach an internal temperature of 160° and 180° F. respectively.

Pork roasts and chops were heated to 170° and 190° F. internal temperature. The manner of cooking was as indicated for the beef. Chops required 15 and 20 minutes and pork roasts 3½ and 4 hours to reach an internal temperature of 170° and 190° F. respectively. As soon as the products had attained the desired temperature, they were immediately placed in No. 10 "C" enamel cans, flushed with nitrogen, sealed, placed in insulated carrying containers, and transported to the Radiation Laboratory where they were placed in the radiation facility. Approximately 8 minutes elapsed between removal of the products from the ranges and placement of the cans in the radiation facility.

Gamma dosages applied were 1.9 and 2.8 megarad. The radiation facility was brought to the same temperatures to which the various meats had been precooked, i. e. 160° to 190° F., depending upon the product and temperature variable.

Precooked, non-radiated control samples were cooled at 34° F. prior to placement in cans; then they were flushed with nitrogen, sealed, and frozen at 0° F. Control samples were returned to our Corvallis laboratory under dry ice whereas the radiated samples were not refrigerated during transit. Upon receipt of these samples, the frozen controls were placed at 0° F. and the radiated samples at 72° F.

Methods of cooking for flavor evaluation. Beef roasts which had not been preheated were cooked in an oven to 180° F. internal temperature as indicated by thermocouples. Pork roasts were cooked to an internal temperature of 185° F. Steaks and bacon were broiled and the chops braised. Salt and pepper were available for judges' use prior to scoring.

Precooked beef and pork roasts were first sliced; then these, as well as the steaks and chops, were preheated to 140° F. in flowing steam. A gravy made from beef or pork stock, flour, seasonings and water, preheated to 190° F. was poured over the products, allowed to drain, and the meat and adhering gravy presented to the judges. The gravy receipe was kept standard throughout the test. The gravy was added primarily for purposes of moistening the precooked and reheated meats.

Flavor evaluation design. For the products which had not been precooked, the hedonic 9-point ballot was used *(6)*. Products evaluated at *like extremely* were marked as 9; those evaluated as *dislike extremely* were scored as 1. The hedonic ballot was modified for the meats which had been precooked prior to radiation. The modification consisted of designating the mid-point of the scale as the reference. Thus, the tasters scored the samples against a reference sample and recorded their judgments on the modified *like-dislike* ballot. The reference sample, included in the array of 7 samples presented to the judges, was non-radiated beef or pork precooked to 180° or 190° F., respectively.

The judges were students at Oregon State College. These students had no previous experience with radiated products. Products were served to the judges in cups coded with 3-digit random numbers. In the case of the ballot in which a reference was used, 7 cups were presented to the taster; one marked "R" (reference) and 6 coded cups representing the 6 treatments.

Subsequent to scoring, the ballots were decoded, scores assigned and analyzed by analysis of variance.

Moisture analysis. Portions of the lean portion of the meat were dried at 158° F. under a 28-inch vacuum to a constant weight. The loss in weight was calculated as % moisture.

Total nitrogen. Total nitrogen, on a moisture-free basis, was determined by the Kjeldahl method *(3)* based on the digestion of the protein with concentrated H_2SO_4 to which a catalyst consisting of 10 g. K_2SO_4 and 0.3 g. $CuSO_4$ had been added. The NH_3 was

distilled into 4% H_3BO_3 containing a mixed indicator and titrated with 0.20 N H_2SO_4. The indicator used was that developed by Ma and Zuazaga *(4)*.

Amino nitrogen. Ten grams of the lean portion of the meat was blended in 100 ml. of water and after filtering, a properly diluted aliquot was analyzed for amino nitrogen by the method of Peters and Van Slyke *(7)* and calculated on a moisture-free basis.

RESULTS AND DISCUSSION

Radiated fresh meats. Mean flavor scores of the beef roasts, pork roasts, beef steaks, pork chops and bacon, prior to storage are shown in Table 1. Flavor panels consisted of 100 to 130 students.

TABLE 1

Mean flavor score of non-radiated and radiated beef steak and roasts, pork chops and roasts, and bacon (Prior to storage)

Treatment	Products				
	Beef Steak	Beef Roast	Pork Chops	Pork Roasts	Bacon
Non-radiated	7.34	7.32	7.12	7.46	6.97
1.9 megarad	6.40	7.00	6.66	6.74	6.15
2.8 megarad	6.15	6.20	5.92	6.41	6.43
L.S.D. (.05)	0.35	0.47	0.39	0.31	0.39

It may be seen from the data (Table 1) that the non-radiated meats were scored significantly higher than the meats radiated at the 2.8 megarad level. Tasters were able to differentiate between the non-radiated and 1.9 megarad levels in beef steak, pork roasts and chops and in bacon. Significant differences in flavor between the radiation levels were shown in beef and pork roasts and in pork chops.

In this laboratory we have used a score of 6.0 (hedonic) as the arbitrary "acceptance" level. In view of this, and considering the data in Table 1, all products at the 1.9 megarad level can be considered as acceptable and at the 2.8 megarad level, beef steak, beef and pork roasts and bacon can be considered acceptable.

If one calculates the per cent of scores above 6.0 as the "per cent acceptance" it is found that, as would be expected, an increase in radiation dosage causes a decrease in the "per cent acceptance". The decrease is greater in some products than in others. The least difference was recorded with beef roasts and pork chops irradiated at the 1.9 megarad level. This decrease was on the order of 7 to 8% (Table 2).

TABLE 2

Per cent judges scoring non-radiated and radiated meats at 6 or above (Hedonic ballot)

Treatment	Beef Steak	Beef Roast	Pork Chops	Pork Roast	Bacon
Non-radiated	90	94	88	93	85
1.9 megarad	75	87	81	78	73
2.8 megarad	60	68	63	75	75

The cans of radiated meats which were placed in storage at 72° F. began to swell within 2 weeks. They did not burst during the storage period. Previous bacteriological analysis on TGY agar had disclosed no viable bacteria

present except in pork roasts where 20 and 3 organisms per gram were observed in the 1.9 and 2.8 megarad levels respectively. The one exception to the swelling of the cans was with bacon. The cans of radiated bacon showed no evidence of spoilage after 2 years at 72° F.

After 9 months in storage, representative cans of these products were opened. No viable organisms could be demonstrated. Fluid loss from the meats was experienced in all products except bacon. This loss ranged from 1 to 10% in the non-radiated, frozen, and thawed samples, to 10 to 25% in the radiated samples stored at 72° F. No difference in fluid loss between the radiation levels could be demonstrated.

All products except bacon had small crystalline deposits of a yellowish-white color at the surface of the meat whether submerged in the liquid extracts or above its surface. These were removed and gave a positive Millon and Ninhydrin reaction. They were insoluble in water but soluble in dilute HCl. When chromatogramed, the spots were typically those of tyrosine. Drake, Giffee, Ryer, and Harriman *(1)* reported the presence of small crystalline deposits of tyrosine in radiated beef which had been stored 3 months at 100° F.

Ratios between total nitrogen and amino nitrogen of the lean portion of the meat are shown in Table 3. A slight change occurred as the result of the radiation treatment but a major change occurred during the storage period. This substantiates the indicated breakdown of the protein which had not been precooked prior to radiation.

The radiated bacon was the only product considered by preliminary examination to be edible after 9 months' storage. Accordingly, the stored bacon was submitted to flavor evaluation panels in the manner previously reported. The non-radiated frozen control was compared to the radiated and stored samples. Results are presented in Table 4.

There were significant changes in the relative scoring of these samples after the storage period. The judges were able to differentiate between the 1.9 and 2.8 megarad levels but not between the non-radiated control and the

TABLE 3

Ratio of total nitrogen/amino nitrogen in lean portions of radiated meats stored 9 months (Moisture-free basis)

Treatment	Beef Steak	Beef Roast	Pork Chops	Pork Roasts
Non-radiated	430	396	517	274
1.9, frozen	383	388	367	269
1.9, stored	67	107	90	113
2.8, frozen	328	294	368	208
2.8, stored	65	96	87	70

TABLE 4

Mean flavor scores of radiated and non-radiated bacon before and after 9 months' storage at 72° F. and 0° F., respectively

	Before Storage	After Storage
Non-radiated	6.97	6.52
1.9 megarad	6.15	6.52
2.8 megarad	6.43	6.08
L.S.D. (.05)	0.39	(0.36)

1.9 megarad level. The percentage scores after 9 months at 6 or above on the hedonic ballot were 77, 77, 67 for the non-radiated, 1.9 and 2.8 megarad samples, respectively.

Meats radiated after precooking. Beef steaks and roasts and pork chops and roasts were evaluated for flavor immediately after radiation and at periods of 80 and 250 days after radiation. The radiated samples were stored at 72° F. while the non-radiated samples were kept at 0° F. These results are presented in Tables 5, 6, 7, 8.

TABLE 5

Mean flavor scores [1] of precooked and radiated beef steak after 80 and 250 days storage at 72° F.

	Initial Evaluation	After 80 days	After 250 days
Non-radiated	5.16	4.89	5.17
1.9 megarad	5.10	4.66	5.20
2.8 megarad	4.64	4.27	4.86
L.S.D. (.05)	0.31	0.27	0.22
No. of tasters	125	138	179

[1] Non-radiated reference sample pegged at 5.0.
[2] Stored at 0° F.

TABLE 6

Mean flavor scores [1] of precooked and radiated beef roasts after 80 and 250 days storage at 72° F.

	Initial Evaluation	After 80 days	After 250 days
Non-radiated [2]	5.00	5.13	5.21
1.9 megarad	4.57	4.82	5.30
2.8 megarad	4.71	4.60	5.17
L.S.D. (.05)	0.23	0.28	NSD
No. of tasters	116	138	179

[1] Non-radiated reference sample pegged at 5.0.
[2] Stored at 0° F.

TABLE 7

Mean flavor scores [1] of precooked and radiated pork chops after 80 and 250 days storage at 72° F.

	Initial Evaluation	After 80 days	After 250 days
Non-radiated [2]	5.22	5.37	5.26
1.9 megarad	5.19	4.18	4.53
2.8 megarad	4.69	3.73	4.40
L.S.D. (.05)	0.32	0.28	0.30
No. of tasters	113	138	178

[1] Non-radiated reference sample pegged at 5.0.
[2] Stored at 0° F.

TABLE 8

Mean flavor scores [1] of precooked and radiated pork roasts after 80 and 250 days storage at 72° F.

	Initial Evaluation	After 80 days	After 250 days
Non-radiated [2]	4.86	4.81	5.14
1.9 megarad	4.98	4.38	5.15
2.8 megarad	4.67	4.23	4.92
L.S.D. (.05)	NSD	0.27	NSD
No. of tasters	102	121	176

[1] Non-radiated reference sample pegged at 5.0.
[2] Stored at 0° F.

The response of the judges to the flavor of the beef steak at the initial period of evaluation disclosed a significant difference in the flavor of the meat which had been precooked and radiated at the 2.8 megarad level. The judges were unable to differentiate between the non-radiated and 1.9 megarad levels. This situation also prevailed at the 80 and 250-day storage period.

Precooked and non-radiated beef roasts were evaluated as being significantly better than the radiated meat at the initial period but after 250 days storage the judges were unable to differentiate between the samples. The judges were unable to distinguish between the pork roasts at the initial and 250-day period of evaluation. There was a slight increase in score during the storage period.

Precooked radiated pork chops reacted somewhat differently. The judges scored the control and the 1.9 megarad samples as not being different at the initial and 80-day periods of evaluation. At the 250-day interval they scored the radiated samples as being signifiantly poorer than the non-radiated control. It is to be noted that at this period, 0.86 units separated the control and 2.8 megarad samples.

The maximum difference between the control and the 2.8 megarad samples occurred in the case of pork chops when evaluated at the 80-day period. This was the only instance wherein the variation between the scores amounted to approximately 2 units. Variation in the scoring of the other products at any period was usually less than 1 unit.

It is to be emphasized that the precooked and radiated products were scored against a reference which was "pegged" at 5 on the modified hedonic scale. This did not allow the judges to exercise "free choice" in their evaluations. However, if the judges are allowed "free choice" in the placement of the evaluation, the score will be approximately 1 to 2 units higher. As an example, the precooked pork roasts were presented to a panel at the 80-day storage interval. The panel was asked to score the control and the 2 radiated samples on an unrestricted hedonic ballot. The mean flavor scores of these samples ranged between 6.3 for the control and 5.4 for the 2.8 megarad level samples. When scored on the restricted "reference" ballot the scores were 4.8 and 4.2, respectively.

In only 3 of the 12 periods of evaluation reported were there any effects of the temperature of precooking employed prior to radiation. These occurred with beef roast at the 80-day storage period wherein the meat cooked to 160° F. was considered better than that precooked to 180° F.; with beef steak, at the initial period, wherein the meat precooked to 180° F. was scored higher than that cooked to 16 ' F., and in pork roast wherein the meat cooked to 190° F. was scored higher than that precooked to 170° F. at the 80-day interval. The magnitude of difference on the reference ballot between the temperatures of precooking was 0.4 units. In no other instance could an effect be demonstrated of the temperatures to which the products were precooked.

All of the precooked meats were firm and there was no evidence of hydrolysis as exhibited by free liquid in the cans, as demonstrated by development of tyrosine crystals, or by changes in the amino nitrogen content of the meat. The amino nitrogen content of the samples, as mg. per g., dry weight basis, varied for each of the products, storage interval and radiation dosage employed. The ranges were 0.8 to 2.1 mg/g. There were no consistent

trends as a result of either the storage temperature, radiation dosage, or duration of storage.

The precooked, radiated beef and pork developed a reddish color. This pigment has been characterized as denatured globin hemochrome by Tappel *(10)*. The reddish color permeated the entire lean portion of the meat but when the meat was sliced and exposed to air, the color soon reverted to a brownish color no different than the color of the non-radiated precooked meat. It did not detract from the appearance of the meat as served to the judges.

SUMMARY AND CONCLUSIONS

Fresh and precooked beef and pork roasts, steaks, chops, and fresh bacon were radiated at levels of 1.9 and 2.8 megarad and stored at 72° F. for periods up to 250 days. Flavor evaluations were made immediately after radiation and at the 80 and 250-day periods.

In radiated fresh meats, extensive degradative changes occurred during the storage period. Excessive fluid loss, the development of tyrosine crystals, and the general breakdown of the protein resulted in meat, though bacteriologically sterile, which was unfit for consumption and no longer acceptable.

Bacon which had received no treatment other than the normal commercial cure and smoke retained its acceptability during 9 months' storage at 72° F.

There was no significant change in the flavor of precooked and radiated beef and pork roasts, steaks and chops during storage at 72° F. for 250 days. No evidence of protein breakdown could be demonstrated and the products were considered acceptable to the judges at the end of the storage period.

On the basis of this experiment, beef cooked to 160° F. and pork cooked to 170° F. prior to radiation will not undergo degradative changes during storage which result in the products being unacceptable to flavor panels.

Acknowledgment

The authors wish to acknowledge the assistance of Major Joseph Berg, Jr. and M/Sgt. Gilbert Jehling, Dugway Proving Grounds, Utah, for their very kind assistance in providing facilities for precooking the meats used in these studies. We wish to acknowledge the assistance of Dr. E. C. Bubl, Agricultural Chemistry, for identifying the tyrosine crystals. To Dr. A. W. Anderson, Bacteriology Department, we express our appreciation for the bacteriological analysis.

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CONTENT OF FREE IMIDAZOLE COMPOUNDS IN THE MUSCLE TISSUE OF AQUATIC ANIMALS*

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(Manuscript received May 12, 1958)

Jones *(11)* recently showed that the muscle tissues of cod contain anserinase, an enzyme which hydrolyzes the dipeptide anserine to its constituent amino acids, l-methylhistidine and β-alanine; and that the enzyme continues to act in sterile cod flesh during storage. Since such a reaction offers promise of developing methods for following fish handling practices independent of microbiological spoilage, it was of interest to us to determine whether anserinase occurs in tuna. Preliminary observations indicated that the various tuna species contained no detectable anserinase activity, but that they did contain unexpectedly large amounts of anserine, approaching in some samples 1% of the wet weight of the tissue. The survey reported in this paper was then undertaken in order to obtain more complete information than was available on the distribution of anserine and related imidazole compounds in the muscles of aquatic animals. Previous work in this field and Yudaev's observations *(30)* in particular were reviewed by Shewan *(18)* in 1951. Subsequent information has been limited to the semi-quantitative observations provided by Shewan *et al. (19)*, and Shewan *(20, 21)*.

The elegant ion-exchange method developed by Davey *(4)* for determining carnosine, anserine, histidine and methylhistidine made possible analyses which had previously been time-consuming and of doubtful accuracy.

EXPERIMENTAL

Materials. The samples of tissue analyzed were obtained from a number of sources. We are indebted to the U. S. Fish and Wildlife Service for frozen samples of several marine and all of the fresh water fish. F. E. Booth Company, Inc., furnished several tuna. One sample of king salmon and one of lingcod muscle were excised and frozen at sea immediately after the fish were caught. The leopard shark was a fresh specimen within a few hours of catch. Fresh whale meat was obtained from the Del Monte Fishing Company. Numerous other samples were obtained from local fish markets.

Preparation of extracts. Five grams of muscle were extracted with 50 ml. of 1% picric acid in a Waring blender as described by Hamilton *(8)*. The extract was filtered (suction) and with the washings was freed of picric acid by passing it through a Dowex-2 column as described by Tallan *et al. (27)*. The effluent was evaporated to small volume in a rotary evaporator, diluted quantitatively to 5 to 25 ml., and either analyzed immediately or held in the frozen state until needed. Those extracts which were not entirely clear after the step of concentration were treated with Celite and refiltered before adjustment to final volume. An extract obtained with 80% alcohol was also found to contain the imidazole compounds quantitatively (Table 2).

Analytical methods. The ion-exchange column procedure of Davey *(4)* was used except that the column length was extended from 50 cm. to 65 cm. Column preparation (Dowex 50-X4) and operation procedures were those of Moore and Stein *(15)*. One ml. fractions were obtained at the rate of 5 per hour with an automatic collector. Elution

* Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 27, 1958.

peaks for histidine, methylhistidine, carnosine and anserine were usually at 50, 56, 80 and 90 ml., respectively. Amino acid and peptide concentrations were determined as described by Yemm and Cocking *(29)* with the following modification suggested by Davey (personal communication). To each 1 ml. of sample add 1 ml. of a 75% methyl cellosolve aqueous solution approximately 0.0002 molar with respect to KCN and 1.15 molar with respect to pH 4.5-5.0 acetate buffer, and 0.2 ml. of a 10% solution of ninhydrin in methyl cellosolve. Heat at 100° for 20 min., cool, dilute with an appropriate amount of 50:50 aqueous alcohol and read optical density at 570 mμ.

Several different techniques were used to identify with certainty the nature of the amino acids and peptides in the extracts. The positions of the elution peaks were determined both by chromatography of known substances alone and in mixtures, and after addition to extracts. DL-Anserine was obtained from General Biochemicals, Inc.; L-carnosine and L-methylhistidine were products of California Foundation for Biochemical Research. Histidine and carnosine were determined quantitatively by the Pauly diazotized sulfanilic acid reaction as well as with ninhydrin. The reagent used was that described by Fraenkel-Conrat and Singer *(5)*. Methylhistidine and anserine give no color. The total optical density obtained with this reagent on the unfractionated extract was in good agreement with the total found for the histidine plus carnosine in the fractions. Histidine decarboxylase (Worthington) was used as recommended by Gale *(6)*. Arginine was estimated in an aliquot of the protein-free extract by the method of Ceriotti and Spandrio *(3)*. Nitrogen was determined by micro-kjeldahl.

RESULTS

In a series of control experiments, the 4 imidazole compounds were added either alone or in mixtures to the column in amounts ranging from 0.3 to 1 μ mole for each. The average recovery was 96% with a range of 78 to 114%. Some data showing the results of duplicate experiments with tissue extracts are shown in Table 1. They indicate that any single determination could be in error by as much as 50%, particularly when only small amounts of the constituents are present. Qualitatively however the

TABLE 1

Duplicate Determinations of Imidazole Compounds in Fish Muscle [1]

	Histidine	L-Methylhistidine	Carnosine	Anserine
	μ mole/g.	*μ mole/g.*	*μ mole/g.*	*μ mole/g.*
Menhaden	18.0	0.3	0.3	0
	12.0	0.3	0.2	0
King Salmon I	1.1	2.4	1.0	14.2
	1.6	1.0	0.6	9.0
King Salmon II	1.1	0	0	16.4
	2.1	0	0	18.6
Humpback Whale	0.8	0.1	22.1	13.2
	0.5	0.1	17.2	10.4
Skipjack	57.3	0	8.0	5.2
	77.3	0	8.9	7.6
Albacore	53.1	0	4.4	10.6
	48.8	0	4.8	11.4
Carp	4.2	0	0.1	0
	3.6	0	0.1	0
Yellow Perch	3.5	0	0.5	0.7
	2.5	0	0.3	0.6

[1] μ Moles per gram wet tissue. 0 signifies < approx. 0.05 μ-moles/gram.

duplicates are in agreement and the method was considered adequate for the survey. Methylhistidine was estimated with difficulty in the presence of large amounts of histidine. The two emerged from the column so close together that a large histidine band tended to obscure a small methylhistidine band.

The adequacy of the methods for histidine and carnosine when they were present in relatively large amounts is shown by a comparison of the results obtained with ninhydrin and with the Pauly reagent (Table 2). Similarly histidine decarboxylase gave results in good agreement with the ninhydrin data (Table 2). Table 2 also records a comparison showing the agreement between colorimetric determination of histidine in the whole extract by the Pauly reagent as compared with ninhydrin color in the histidine fraction from the column, and a comparison of the results obtained by 80% alcoholic as well as by picric acid extraction.

TABLE 2

Comparison of Methods for Determining Histidine and Carnosine [1]

	Histidine			Carnosine	
	Ninhydrin	Pauly	Decarboxylase	Ninhydrin	Pauly
Skipjack	72.7		68.1 [2]		
Skipjack	57.3	62.5		8.0	7.3
Barracuda	8.4		6.8 [2]		
Sardine [3]	46.0	45.9 [2]			
Sardine [4]	47.8	48.7 [2]			

[1] μ-moles/gm. wet tissue. On column-separated fractions except as noted.
[2] On unfractionated extract.
[3] Picrate extract.
[4] 80% alcohol extract.

A tabulation of the analyses is presented in Table 3. It should be emphasized that a number of samples, particularly those that contained only small amounts of imidazole compounds, were analyzed once and therefore that much of the data has qualitative significance only. In general, members of the tuna family had the highest total content of imidazole compounds, as anserine and histidine in approximately equal parts. In some samples these two compounds accounted for over 50% of the total non-protein nitrogen. Light muscle was higher in imidazole content than red muscle. Other fish with relatively high amounts of total imidazole contained mostly anserine (salmon, sable and swordfish) or histidine (mackerel, sardine, and menhaden) but not both. Barracuda, shad, and one carp contained intermediate amounts of histidine. Methylhistidine for the most part was absent. It is believed that its presence reflects anserinase activity *(11)*, although further work will be required to prove this. Only the true cod and two species of salmon showed appreciable amounts. Absence of methylhistidine from all of the tuna samples is indicative of the lack of anserinase.

Carnosine is the most important imidazole in the whales. In this respect the whale resembles the ox and the horse, but differs from other mammals *(28, 31)*. Our data for sperm whale confirm those of Davey *(4)*. Presence of even small amounts of carnosine in other oceanic species was somewhat unexpected in view of the belief that it was not present at all *(18, 30)*. Presumably, techniques hitherto used were not of sufficient precision to show carnosine unequivocally. In the present work, small amounts occurred in all the mollusca and crustacea samples but of these only the oyster contained anserine.

Most of the extracts were also assayed for arginine in the thought that this amino acid might be present if the imidazole compounds were not. Less than 1 μ mole/g. wet tissue was present in all except crab, shrimp and squid which contained 4.0, 1.6 and 1.4 μ mole/g., respectively.

DISCUSSION

Zapp and Wilson *(31)* have called attention to the considerable variations of carnosine and anserine content within a given mammalian species. The imidazole content differed according to the muscle type (more in light than in dark muscle) and with the same muscle in different members of the

TABLE 3

Histidine, 1-Methylhistidine, Carnosine, Anserine and Non-protein Nitrogen Content of Muscle of Aquatic Animals

Species	Sample	Histidine	L-Methylhistidine	Carnosine	Anserine	Non-protein Nitrogen (NPN)	Imidazole N in NPN
		μ mole/g.	μ mole/g.	μ mole/g.	μ mole/g.	mg./g.	%
Marine Fish							
Yellowfin tuna (*Neothunnus macropterus*) (light meat)	1	35.5	0	0.3	29.9	5.0	63
Yellowfin tuna (light meat)	2	47.4	0	0	30.7	6.2	60
Yellowfin tuna (light meat)	3	36.8	0	0	41.6	6.5	60
Yellowfin tuna (light meat)	4	26.6	0	0.1	25.5	10.2	26
Yellowfin tuna (light meat)	5	47.7	0	5.0	19.1	6.6	51
Yellowfin tuna (dark meat)	5	13.7	0	2.3	9.3	4.4	28
Albacore (*Thunnus germo*) (light meat)	1	39.1	0	1.2	38.9	10.7	36
Albacore (light meat)	2	50.0	0	4.4	10.6	3.7	80
Albacore (dark meat)	2	17.5	0	0.6	13.7	2.9	53
Skipjack (*Katsuwonus pelamis*) (light meat)	1	76.8	—	1.9	24.5	—	—
Skipjack (light meat)	2	26.0	—	1.6	19.8	—	—
Skipjack (light meat)	3	57.3	0	8.0	5.2	7.4	43
Skipjack (dark meat)	3	11.1	0	2.6	1.3	3.7	19
Bigeye tuna (*Parathunnus mebachi*) (light meat)	1	30.5	0	0	27.9	5.1	55
Bigeye tuna (light meat)	2	31.5	0	0	31.7	6.4	48
Mackerel (*Pneumatophorus diego*)	1	38.7	0	0.2	0.7	3.7	45
Mackerel	2	33.5	0	0.6	0.2	4.2	35
Sablefish (*Anoplopoma fimbria*)		2.1	0	1.4	18.4	2.7	45
Swordfish (*Xiphius gladius*)		0.2	0	0.5	15.5	2.4	38
Sardine (*Sardinops caerulea*)		49.2	0	0.1	0.3	4.8	44

King salmon *(Oncorhynchus tshawytscha)*	1	1.4	1.7	0.8	11.6	2.9	29
King salmon	2	2.4	0	1.2	19.9	3.0	43
King salmon	3	1.6	0	0	17.5	4.9	22
Pink salmon *(Oncorhynchus gorbuscha)*	4	2.6	0	0	23.0	2.1	67
Silver salmon *(Oncorhynchus kisutch)*	5	3.1	1.5	0.5	25.5	4.4	37
Menhaden *(Brevoortia tyrannus)*		15.0	0.3	0.3	0	3.8	17
Shad *(Alosa sapidissima)*		7.3	0	0.4	0	2.2	15
Cod *(Gadus sp.)*		0.2	1.2	0.2	5.0	2.8	13
Barracuda *(Sphyraena argentea)* (light meat)	1	8.4	0	0.1	0.1	2.9	12
Barracuda (dark meat)	1	3.3	0	0.2	0	2.1	7
Halibut *(Hippoglossus stenolepsis)*	1	0.3	0	0.5	3.7	3.8	7
Halibut	2	0.2	0	0.3	1.5	1.8	6
Rockfish *(Sebastodes sp.)*	1	0.3	0	0	0	2.9	0.4
Rockfish	2	0.6	0	0.2	0	2.4	1.1
Lingcod *(Ophiodon elongatus)*	1	0.2	0	0	0	3.1	0.3
Lingcod	2	0.3	0	0.2	0.3	2.5	1.6
Ocean perch *(Sebastodes alutus)*		0.5	0	0.3	0.2	2.9	1.7
Smelt *(Atherinopsis californiensis)*		0.1	0	0.2	1.5	2.5	0.4
Sole *(Glyptocephalus zachirus)*		0.8	0	0.3	0.3	3.1	2
Sea bass *(Stereolepsis gigas)*		0.3	0	0	0	1.6	0.6
Butterfish *(Isopsetta isolepsis)*		0.2	0	0	0	2.0	0.5
Skate *(Raja inornata)*		0	0.1	0.3	0	12.5	0.2
Leopard shark *(Triakis semifasciata)*		0	0	0.1	0	11.1	0.1
Fresh Water Fish							
Sheepshead *(Aplodinotus grunniens)*		3.2	0	0.9	0	2.3	8
Rainbow trout *(Salmo gairdnerii)*		2.5	0	2.1	0.4	3.8	16
Buffalo *(Ictiobus sp.)*		2.4	0	0.7	0	2.2	6
Carp *(Cyprinus carpio)*	1	3.9	0	0.1	0	3.5	5
Carp	2	10.1	0	0.3	0	2.9	15
Yellow perch *(Perca flavescens)*		3.0	0	0.4	0.7	2.7	7
Yellow pike *(Stizostedion vitreum)*		4.0	0	0.4	3.5	2.2	17
Mollusca and Crustacea							
Squid *(Loliqo opalescens)*		1.3	0.2	0.5	0	6.3	0.2
Crab *(Cancer magister)*		0.2	0	0.2	0	4.6	0.4
Shrimp *(Pandalus borealis)*		1.2	0.2	2.0	0	5.5	3
Scallop *(Pecten sp.)*		0.3	0	0.3	0	2.4	1
Abalone *(Haliotus sp.)*		0.3	0	0.5	0	—	—
Oyster *(Crassostrea gigas)*		0.5	0	0.2	1.6	2.4	7
Marine Mammals							
Humpback whale *(Megaptera nodosa)*		0.7	0.1	20.0	11.8	5.3	34
Sperm whale *(Physeter catodon)*		0	0	5.2	1.9	3.3	12

same species. They also noted that a muscle in general contains more of one imidazole than the other, the same one being in excess for all skeletal muscles of a given species.

In general, our data are in agreement with these conclusions. There was more anserine than carnosine in the marine fish, but in the fresh-water fish, the whales, and the invertebrates examined, except the oyster, carnosine was present in higher concentrations. Carnosine was also present in higher concentration than anserine in a single specimen of skipjack (skipjack 3, Table 3).

The variable content of carnosine and anserine in the same muscle of different individuals of the same specie can possibly be accounted for on the basis of differences in diet, exercise, stage of maturity, or, less likely, to pathological conditions, i.e., muscle disease, and perhaps to chemical individuality. Thus, Hunter *(9, 10)* observed that the carnosine content of the gastrocnemius muscle of cat could be decreased by fasting the animal or by preventing use of the muscle and could be increased by liberal feeding of meat. Koldaev *(14)* obtained similar evidence. The diet effects were probably due to a deficiency or surplus in dietary histidine. Histidine and carnosine contents were markedly lowered, but the anserine level was unchanged in Chinook salmon fingerlings which were grown on a histidine-deficient diet. [b]

Severin and Yudaev *(16)* followed the changes in carnosine and anserine contents of skeletal muscle of rabbits with age. Carnosine first appeared on the 24th day (23 mg%) of the embryonic development, increased in young rabbits to a maximum of about 190 mg%, and then declined to about 70 mg% in adult rabbits. Anserine first appeared on the 7th day and gradually increased to about 450 mg% in adult rabbits. Young rooks contained considerable carnosine but no anserine, whereas, in the adults, the carnosine content was low and the anserine content was high. Carnosine and particularly anserine were decreased in concentration in the muscles of vitamin E-induced muscular dystrophic rats *(17)*. Similarly, L-methylhistidine, carnosine and anserine were decreased in concentration in the muscles of severely dystrophic rabbits, although the levels of most of the amino acids were elevated *(26)*.

No clear picture of the role of the free imidazole compounds emerges from these observations. Bate-Smith *(2)* has suggested that they act in a buffering capacity. Several reports, particularly in the Russian literature (reviewed by Stekol, *23)*, suggest that they may contribute to the metabolic activity of the muscle *(24, 25)*, possibly via phosphate derivatives *(7)*. It has been suggested that the imidazole compounds are involved in flavor *(1, 22)*.

The presence of relatively large amounts of histidine in tuna, mackerel and sardine has been known for some time (reviewed by Shewan, *18, 21)*. Free histidine is undoubtedly the source for the relatively large amounts of histamine which occasionally cause outbreaks of food-poisoning after ingestion of some types of spoiled fish (reviewed by Shewan, *21)*. The highest amount of histidine previously reported was 400 mg. histidine nitrogen per

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[b] Unpublished, this laboratory. Experimental fish, courtesy of Dr. J. Halver, U. S. Fish and Wildlife Service, Cook, Washington.

100 g. of flesh in mackerel *(12, 13)*. The value for mackerel obtained in this study was 136 mg. histidine nitrogen per 100 g.

In line with Shewan's observations *(21)*, the pelagic fish contained relatively high amounts of imidazole compounds as compared with flat fish, fresh water fish, or elasmobranchii, but no attempt will be made here to correlate the compositional studies with species.

SUMMARY

An ion-exchange column method has been used to survey the amounts of anserine, carnosine, histidine and L-methyllhistidine in the muscle tissues of 23 species of marine fish, 2 elasmobranchii, 6 fresh water fish, and 6 molluscs and crustacea. Relatively large amounts (10 to 50 μ moles/g. each) of anserine and histidine were found in the four tuna species investigated. The salmon, swordfish and sablefish had large amounts of anserine only (11 to 25 μ moles/g.). The mackerel, sardine, and menhaden contained large amounts of histidine only (15 to 49 μ moles/g.). Light meat of these fish contained more imidazole compounds than did the dark meat. Large variations in imidazole content were noted with different individuals of the same species. Some of the implications of these findings are discussed.

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VARIATION IN COMPOSITION OF SOUTHEASTERN ALASKA PINK SALMON[a]

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Since Green *(2)* conducted his pioneer work on the storage of fat in the muscular tissue of king salmon in 1913, interest has increased in the variation of oil content in salmon. The way in which the protein and moisture vary in relationship to the amount of oil is also of interest. In addition, the emphasis physicians and dieticians now are placing on low-sodium diets in treating various disorders, as well as the increasing importance being assigned to the sodium-potassium balance in normal body functioning necessitates the determination of these elements. Thus, it was found desirable to analyze for the six constituents: moisture, oil, protein, ash, sodium, and potassium. Attention is focused on pink salmon *(Oncorhynchus gorbuscha)* because it is one of the more abundant of the Pacific Ocean species of salmon. The present study was conducted in Ketchikan, Alaska, since there were no pink salmon in Washington coast waters during the time available for the study.

EXPERIMENTAL

Collection of specimens. A thorough investigation of composition should include variation due to: (1) section of the fish from which the sample was taken, (2) sex, (3) size, (4) degree of maturity, (5) season of capture, (6) geographical area of capture, and (7) the population[b] of fish. A beginning was made on the study of some of these variables during the summer of 1956 by collecting 3 male and 3 female specimens each, at the opening, middle, and close of the commercial fishing season. All of these fish were taken by gill net in either Burroughs Bay or near-by Herman Creek, northeast of Ketchikan. Samples were prepared from 7 different sections of each fish and analyzed for the 6 constituents listed above.

All samples were obtained from cannery tenders in Ketchikan, and had been caught the previous night. Thus, they were received within 24 hours of capture. Five female and 5 male fish were collected in each of the 3 series as follows: (A) on July 16, 1956, the first day of the commercial season; (B) on August 2, near the middle of the season; and (C) on August 15, the last day of the season. Because all 10 of the fish in series C proved to be males, it was necessary to secure additional females. These, called series D, were obtained by the U. S. Fish and Wildlife Service vessel, *Sable Fish*, from Herman Creek, west of Burroughs Bay. They were captured August 19, and received in Ketchikan on August 20. The 3 best-appearing fish of each 5 in each series were selected for processing, giving a total of 21 specimens, of which 9 were females and 12 were males.

Preparation of samples. The fish were washed, measured, and weighed as soon as they were received at the laboratory. Each fish was dipped in boiling water for a few seconds, to loosen the skin, and the skin then was peeled off. After the fish had been eviscerated and decapitated, 3 one-inch steaks were removed from the nape (n), center (c), and tail (t) sections (see Figure 1). From the remaining 2 portions labeled (LM), 4 more samples were prepared from portions labeled as follows: the dorsal fatty layer (d) along the back, the dark meat (l) along the lateral section of both sides, the belly flaps

[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 27, 1958.

[b] Pink salmon mature in a two-year cycle of even- and odd-year populations. Although both populations are found in Alaska, only the odd-year population is found in Washington.

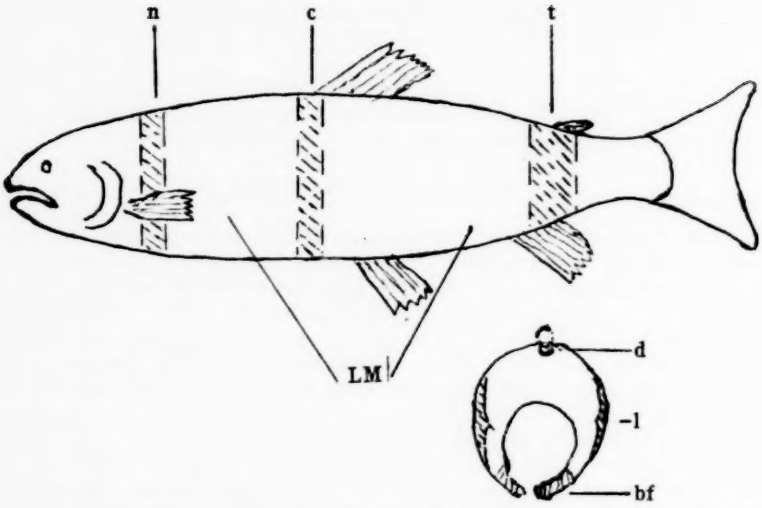

Figure 1. d—dorsal; l—lateral; bf—belly flaps; n—nape; c—center; t—tail; LM—main parts after sections removed; lm—remaining light meat.

(bf), and the remaining light meat (lm). Each sample was weighed, ground in a Hobart grinder, vacuum-packed in a ½-pound can, and stored at − 28° C. until analyzed. Series A was analyzed in Ketchikan. The remaining series were shipped to Seattle and stored at − 18° C. until analyzed.

Methods of analysis. Standard Methods of Analysis of the Association of Official Agricultural Chemists *(1)* were used to determine the percentage of moisture, oil, protein, and ash in each sample.

Sodium and potassium values were determined in units of mg./100 g. by means of a direct-reading flame photometer, using an adaptation of the procedure developed by the National Canners Association Research Laboratory *(3)*.

RESULTS AND DISCUSSION

The 21 specimens examined showed wide variation in composition (Table 1). The total liquid (moisture plus oil), however, ranged in most cases only from 79 to 83%, with an average of about 81%. The greatest variations in moisture were found in the dorsal samples and ranged from 44 to 84%. These figures were not actually determined, owing to insufficient sample to run a moisture determination, but the moisture was obtained by subtracting the sum of the 3 components determined (protein, oil, and ash) from 100%. Thus, the factor of variation for moisture is nearly 2. For the values actually determined, the moisture varied from 63.1 to 80.2%. The greatest variation in composition was found in the oil component. It ranged from 1.3% in the light meat to 43.3% in the dorsal, giving a factor of variation of 33. The protein content showed a rather surprising variation from 9.6[e] to 22.0%, giving a factor of variation of 2.3. The ash varied from 0.75 to 1.68%. Here

[e] This is the lowest content of protein for salt-water fish so far determined in this laboratory. The liquid content (moisture plus oil) constituted almost 90% of this particular fish, which also is the highest value thus far obtained.

TABLE 1

Composition of Southeastern Alaska pink salmon (Oncorhynchus gorbuscha)

Part	Moisture		Oil		Protein		Ash		Sodium		Potassium	
	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range	Avg.	Range
	%		%		%		%		Mg./100 g.		Mg./100 g.	
A Males												
Dorsal	(70) [1]	(44)-(84) [1]	14.7	3.4-43.3	12.5	9.6-15.6	0.91	0.75-1.04	127	80-198	168	123-205
Lateral	70.5	65.7-72.5	11.7	8.1-16.6	18.0	15.4-19.1	1.24	1.01-1.54	67	55- 78	240	187-297
Belly flaps	73.3	66.8-79.5	9.5	3.1-15.4	16.9	15.6-18.3	0.93	0.81-0.98	105	89-130	204	173-226
Nape	76.1	73.5-77.9	4.5	1.8- 7.0	18.9	17.3-20.2	1.10	0.97-1.19	86	70-108	262	230-273
Center	76.2	73.9-77.8	3.4	1.7- 5.4	19.9	17.9-22.0	1.16	1.04-1.33	75	59- 94	282	257-304
Tail	77.6	75.5-78.4	2.4	1.8- 3.5	19.9	18.9-21.8	1.14	1.03-1.21	85	64- 96	279	253-296
Average	76.6		3.4		19.6		1.13		82		273	
Light meat	77.3	75.6-78.4	2.0	1.3- 3.9	20.4	18.8-21.6	1.16	1.09-1.30	71	53- 88	292	264-310
B Females												
Dorsal			17.1	2.1-39.8								
Lateral	69.4	63.1-75.4	13.3	7.8-18.5	16.8	15.6-18.2	1.13	0.99-1.21	58	51- 70	232	208-257
Belly flaps	72.5	66.2-80.0	9.4	2.2-17.8	17.2	16.0-19.1	1.00	0.91-1.06	89	67-126	225	197-271
Nape	75.5	71.6-80.1	5.1	2.0- 9.1	18.7	17.0-19.9	1.21	1.10-1.27	66	52- 76	281	245-334
Center	76.1	73.5-79.2	3.6	1.8- 5.4	19.6	18.2-21.3	1.24	1.10-1.34	58	46- 70	306	252-351
Tail	77.0	74.7-79.2	2.8	1.8- 3.6	19.9	18.6-21.6	1.21	1.06-1.40	67	60- 77	282	218-324
Average	76.2		3.9		19.4		1.22		64		290	
Light meat	77.6	75.6-80.2	2.3	1.4- 3.6	20.3	18.1-21.5	1.37	1.12-1.68	57	46- 76	306	290-343
C All specimens												
Dorsal [2]	(70) [1]	(44)-(84) [1]	15.7	2.1-43.3	12.5	9.6-15.6	0.91	0.75-1.04	127	80-198	168	123-205
Lateral	69.9	63.1-75.4	12.5	7.8-18.5	17.5	15.4-19.1	1.20	0.99-1.54	63	51- 78	238	187-297
Belly flaps	72.9	66.2-80.0	9.5	2.2-17.8	17.0	15.6-19.1	0.98	0.81-1.06	99	67-130	212	173-271
Nape	75.9	71.6-80.1	4.8	1.8- 9.1	18.8	17.0-20.2	1.13	0.97-1.27	78	52-108	270	230-334
Center	76.2	73.5-79.2	3.5	1.7- 5.4	19.8	18.2-22.0	1.19	1.04-1.34	68	46- 94	292	252-351
Tail	77.2	74.7-79.2	2.6	1.8- 3.6	19.9	18.6-21.8	1.17	1.03-1.40	78	60- 96	280	218-324
Average	76.4		3.6		19.5		1.16		75		281	
Light meat	77.4	75.6-80.2	2.1	1.3- 3.9	20.4	18.1-21.6	1.25	1.09-1.68	65	46- 88	298	264-343

[1] ( ) = Values not determined, calculated from others (see text).
[2] Except for oil values, values are for males only.

the factor of variation was 2.2. The high values were obtained for samples whose ash remained dark in color even after heating for 24 hours at 550° to 580° C. Sodium showed a factor of variation of 4.3 with a low content of 46 and a high content of 198 mg./100g.[4] Potassium had a smaller variation with a factor of 2.8. The low content was 123, and the high 351 mg./100 g.

The way in which the constituents varied in relationship to one another (Table 1) is interesting. As would be expected, the moisture varied inversely with the oil. The greatest fluctuation was found in the dorsal, lateral, and belly flap parts, which are only a minor portion of the edible flesh. The steak sections, which make up the usual food serving, were relatively low in oil, and the amount decreased from nape to tail in the range from 5 to 3%. These low values of oil were accompanied by corresponding increases in the amount of moisture.

Protein percentages showed some variation with respect to moisture and oil but there was no uniformity. There appeared to be a direct relationship, however, between the amount of protein and of potassium found in the flesh. The fluctuations were great and the number of samples small, but the averages were as follows:

All Protein Percentages	All Potassium Values (mg./100 g.)	
	Average	Range
Below 16.0	188	160 - 203
Between 16.0 & 20.0	274	210 - 324
Above 20.0	312	290 - 351

In many cases, the amount of sodium seemed to vary inversely with the amount of ash. Again the fluctuations were great and the number of samples small, but the averages indicated a possible inverse correlation:

All Ash Percentages	All Sodium Values (mg./100 g.)	
	Average	Range
Below 1.0	108	70 - 152
Between 1.0 & 1.2	80	51 - 126
Above 1.2	61	46 - 78

It is recognized that, at present, these variations should be considered merely as trends, the verification of which will depend on results from a wider variety of samples that will be analyzed in the future. Unpublished results from a 1957 series of Puget Sound pink salmon, however, gave essentially the same comparisons.

The variation of constituent percentages in regard to their position in the fish also was interesting, and in some cases surprising. This was especially true in the case of oil. The dorsal part had the highest percentages as well as the greatest variation (2.1 to 43.3) (Table 1). The belly flaps ranged from 2.2 to 17.8% fat, and frequently were lower in this constituent than was the lateral muscle, which had a range of 7.8 to 18.5%. The other parts of the

[4] It is important to note that values above 100 mg. were found only in the dorsal and belly flap parts, which constitute but a minor part of the total edible portions of the fish.

TABLE 2

Comparative data of Southeastern Alaska pink salmon [1] (Oncorhynchus gorbuscha)

Section	Moisture	Oil	Protein	Ash	Sodium	Potassium
	%	%	%	%	Mg./100 g.	Mg./100 g.
A. Males — small size, sexually immature						
Lateral	68.3	13.2	18.4	1.22	69	230
Belly flaps	68.6	13.2	17.3	0.90	93	200
Steaks	75.5	3.8	20.7	1.14	84	269
Light meat	76.4	2.2	21.2	1.14	75	268
Average	72.2	8.1	19.4	1.10	80	242
Females — small size, sexually immature						
Lateral	65.9	16.2	17.5	1.17	53	231
Belly flaps	67.3	14.1	18.0	1.01	68	223
Steaks	74.0	4.9	20.6	1.29	61	281
Light meat	76.3	2.5	21.2	1.52	47	311
Average	71.2	9.4	19.4	1.33	57	261
B. Males — small size, medium sexual maturity						
Lateral	72.5	10.3	18.6	1.24		
Belly flaps		4.9	15.6	0.96		
Steaks	77.0	3.0	19.4	1.20		
Light meat	77.8	1.8	20.4	1.21		
Average	75.8	5.0	18.5	1.15		
C. Males — large size, sexually mature						
Lateral	71.8	8.9	18.8	1.41	74	282
Belly flaps	78.0	4.3	16.6	0.98	124	212
Steaks	78.0	2.0	19.6	1.10	80	281
Light meat	78.3	1.4	20.2	1.12	80	299
Average	76.5	4.2	18.8	1.15	92	269
Females — large size, sexually mature						
Lateral	74.5	8.8	15.9	1.10	68	243
Belly flaps	79.6	3.3	16.4	1.03	123	206
Steaks	79.0	2.3	18.2	1.13	71	280
Light meat	80.1	1.7	18.2	1.18	69	304
Average	78.3	4.0	17.2	1.11	83	258
D. Males — large size, most sexually mature						
Lateral	72.3	8.2	18.9	1.54	78	254
Belly flaps	79.5	3.1	17.2	0.98	121	218
Steaks	78.0	1.8	19.7	1.08	95	271
Light meat	78.3	1.3	20.3	1.12	88	290
Average	77.1	3.8	19.0	1.18	96	258

TABLE 2 (continued)

Section	Moisture	Oil	Protein	Ash	Sodium	Potassium
	%	%	%	%	Mg./100 g.	Mg./100 g.
Females — large size, most sexually mature						
Lateral		7.8				
Belly flaps	79.8	2.2	16.9	1.06		
Steaks	78.9	1.9	18.5	1.13		
Light meat		1.7				
Average		3.4				

[1] To conserve space dorsal values have been omitted since data were available for a small part of the constituents; and nape, center, and tail sections have been combined under the heading "steaks".

fish were generally quite low in oil, ranging from 1.3 to 9.1%. The dorsal and the belly parts lost most of their oil at maturity, whereas the lateral muscle usually lost less than half of its oil. In the steak sections (nape, center, and tail), the moisture increased and the oil decreased from nape to tail. In the center part, the sodium was lower than in the nape or tail.

The variation in composition with time of capture was not great, since the commercial season covers a period of only 4 weeks in Alaska. The variation in condition seemed to be more important than either seasonal or size variation. A wide variation in size of specimens was obtained, ranging from 1.37 Kg. to 3.04 Kg. This range was quite large for pink salmon, a relatively small member of the salmon family. Although no large prime* specimens were obtained, it appears that the difference between small and large prime specimens of the same degree of maturity were less than were found in the case of prime and of mature specimens regardless of size (Table 2). Small prime specimens had about the same composition whether they were male or female, except that the female had the higher content of oil and lower content of sodium. In the case of large mature specimens, however, the female had the lower oil content. The variation with maturity was very pronounced and was greatest in the case of oil content. Comparisons of large and small males and females showed about the same changes, with a great variation in content of oil, especially in the dorsal and belly parts, and with a lesser variation in the content of protein. Since the smaller specimens were generally in good condition and the larger ones were about ready to spawn, the importance of condition rather than size is emphasized. In the pink salmon that were about to die, nearly all the oil had been consumed except in the lateral part, which had lost about half of its maximum amount. The amount of protein decreased, and the amount of moisture increased. The variation in oil noted here is in agreement with the work of Green on the lateral muscle of king salmon. He concluded that the lateral muscle was the main depot of storage for fat and that this muscle retained most of its supply in the mature fish.

SUMMARY

Chemical analyses of various sections of large and small, male and female pink salmon captured during the opening, middle, and close of the 1956 commercial fishing season in Ketchikan, Alaska, support the following conclu-

* By prime is meant a specimen in best condition physically, but not sexually mature.

sions: (a) there is great variation in composition in the various sections of each specimen, (b) the greatest variation is in oil content, with the highest values being found in the dorsal, lateral, and belly flaps, in decreasing order, (c) the oil content varies inversely with moisture, (d) a high sodium content is associated with a low content of ash, and a high potassium content is associated with a high content of protein, (e) variations with sex and size are small in comparison with variations due to sexual maturity.

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CHARACTERIZATION OF THE RED PIGMENT FORMED IN THE 2-THIOBARBITURIC ACID DETERMINATION OF OXIDATIVE RANCIDITY [a, b]

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The red pigment formed in the reaction between 2-thiobarbituric acid (TBA) and oxidized lipids has been used as an empirical measure of fat deterioration by many workers in a wide variety of food products *(2, 4, 11, 12, 15, 17, 18, 19, 20)*. Kohn and Liversedge *(8)* suggested that the TBA reactive material was a carbonyl compound since the reaction was blocked by semicarbazine or phenylhydrazine. Bernheim, Bernheim, and Wilbur *(1)* reported the isolation of an impure red TBA pigment and suggested that the reactive compound was a 3-carbon chain containing one oxygen atom. Patton and Kurtz *(12)* tested a number of substances and concluded on the basis of spectral measurements that malonaldehyde was responsible for the TBA pigment and that this compound existed in rancid milk fat. Patton, Keeney and Kurtz *(11)* in a paper regarding the Kreis color reaction for rancid fats pointed out the similarity between epihydrin aldehyde and malonaldehyde. They reported that the TBA reactive material from oxidized milk fat was a water soluble, low molecular weight, Kreis positive, carbonyl compound similar to malonaldehyde. In addition, they postulated that malonaldehyde would be strongly acidic, enolic, and relatively stable on heating with dilute mineral acids. These properties are not expected of epihydrin aldehyde but would be of malonaldehyde. Kurtz, Price and Patton *(9)* suggested that the oxidation product of milk fat and malonaldehyde was the same and theorized that the reaction with TBA occurs by attack of the monoenolic form of malonaldehyde on the active methylene groups of TBA, followed by ring closure.

Dox and Plaisance *(5)* reported the reaction between TBA and a number of aromatic aldehydes to form highly colored derivatives. Kohn *(7)* suggested that TBA was a specific reagent for sulfadiazine and that the reaction involved the amino pyrimidine ring. Shepherd *(16)* in an extensive study of the reaction between TBA and pyrimidines established that TBA was specific for the pyrimidine ring providing there were no substituents in the 4-, 5-, or 6-position. The rate of color formation varied greatly with the various functional groups on the pyrimidine ring, but the molecular extinction coefficients of the reaction product were the same. Jennings, Dunkley and Reiber *(6)* reported that although the pigments prepared from oxidized milk fat, malonaldehyde and sulfadiazine exhibit the same spectral characteristics in the visible range, that did not justify the conclusion that the compounds

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[b] Presented at the 18th Annual Meeting of the Institute of Food Technologists, Chicago, May 28, 1958.
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are identical. Their study did not report any definite conclusion regarding the identity of the pigments, the structure, or the reaction by which they are formed. A spectrophotometric technique suggested that the malonaldehyde TBA pigment was formed as an equi-molar reaction without loss of water.

In 1955, Patton and Kurtz *(13)* reported that freshly prepared α or β unsaturated aldehydes did not give the TBA test but when they were autoxidized or heated with Cu^{++} ions a positive reaction resulted. Recently, Sinnhuber and Yu *(18)* described a method for the quantitative determination of malonaldehyde with TBA using 1, 1, 3, 3-tetraethoxypropane (TEP) as a standard. Acid hydrolysis of TEP yields malonaldehyde, which reacts with TBA to yield the red pigment with an absorption maximum at 535 mμ. The results were expressed as TBA number, or milligrams of malonaldehyde per 1000 g. of sample. No evidence was offered, however, that the free malonaldehyde existed in rancid fishery products.

This paper describes the preparation and isolation of the TBA pigment from rancid salmon oil, 1, 1, 3, 3-tetraethoxypropane (TEP) and sulfadiazine as the pure, crystalline compound. Although prepared from widely diverse materials, elemental chemical analysis, absorption spectroscopy, combining ratios, and paper chromatography strongly indicate that all the pigments are identical. The equation for the formation of the pigment and a proposed structural formula are presented.

EXPERIMENTAL METHODS AND RESULTS

Preparation of pigments. *Preparation of Pigment from 1, 1, 3, 3-tetraethoxypropane (TEP).* The procedure used was a modification of one described by Dox and Plaisance *(5)* for the preparation of aromatic aldehyde derivatives of TBA. TBA (0.0125 mol.) and TEP (0.00625 mol.) were refluxed with 250 ml. of 12% hydrochloric acid in a boiling water bath for 90 minutes. The pigment separated from the acid solution as dark purple-black needles. Crystals were filtered on a sintered glass filter funnel with suction; washed with 100 ml. of 0.6N hydrochloric acid; briefly with hot water; then with 20 ml. of ethyl alcohol; with 100 ml. of 1:1 alcohol and ethyl ether and finally with 100 ml. of ethyl ether. Since TBA is slightly soluble in ethyl ether, it may be removed by continuous washing. The purple needles were dried in a vacuum oven at 60° C. The yield obtained was 91%, assuming a 2:1 mole reaction with the loss of 2 mols of water.

In another preparation the methyl compound, 1,1,3,3-tetramethoxypropane, was used. The yield and results obtained were the same.

Preparation of Pigment from Sulfadiazine. Sulfadiazine (0.02 mol.) and TBA (0.04 mol.) were refluxed with 350 ml. of 12 percent hydrochloric acid for 90 minutes in a boiling water bath. The dark purple needle-like crystals were filtered on a sintered glass filter funnel and washed as described in the preparation of pigment from TEP. The purple crystals were dried in a vacuum oven at 60° C.

Preparation of Pigment from Rancid Salmon Oil. A 3-liter flask containing 1000 g. of rancid salmon oil with a TBA number of 257 *(18)* and 20 ml. of 1:1 hydrochloric acid were heated on a steam bath. A stream of nitrogen served to stir the mixture and carry the more volatile material through a reflux condenser (water temp. 13° C.) A delivery tube connected to the top end of the condenser passed the volatiles to a flask containing 250 ml. of a 1% TBA solution in 0.6N hydrochloric acid. After 2½ hours the flask containing the acid TBA solution was heated on a boiling water bath under a reflux condenser for 40 minutes. Separation of the crystalline pigment was carried out as described above in the preparations using TEP and sulfadiazine.

Separation of Free Malonaldehyde from Rancid Salmon Oil. Existence of malonaldehyde as the free aldehyde in rancid products has never been demonstrated. This highly reactive material may exist as an acetal as suggested by Powick *(14)* It was noticed that this rancid salmon oil had a sharp penetrating odor similar to formaldehyde, but this may be caused by other carbonyls. This experiment was an attempt to answer the question of the presence or absence of free malonaldehyde in rancid fat. Ten grams of salmon oil

(TBA number 257) were held at room temperature while a stream of nitrogen carried any volatile material through a reflux condenser (water temperature 13° C.) and into a flask containing a solution of TBA in 0.6N hydrochloric acid. After 2½ hours, a quantitative determination of malonaldehyde was carried out on the TBA solution *(18)*. Results obtained indicate that a small amount of malonaldehyde, 2.2% of the total amount, probably was present as the free carbonyl.

Purification of the Pigments for Elemental Analyses. One gram of pigment, prepared as described above, was finely ground in a mortar. The powder was boiled for 40 minutes with 200 ml. of 0.6N hydrochloric acid, cooled to 60° C. and filtered on a sintered glass funnel with suction. The powder was washed with 100 ml. of 0.6N hydrochloric acid; 25 ml. of cold water from a wash bottle; 25 ml. of ethanol in the same manner; and finally with 100 ml. of diethyl ether. The dark purple powder was dried in a vacuum oven at 60° C. to constant weight.

Absorption spectrophotometry. The 3 pigments described above were compared in a Beckman DU spectrophotometer. Solutions of the pigments were made by carefully weighing 10 mg. samples, dissolving the pigments in 10 ml. of 0.1N sodium hydroxide, adding distilled water, then 10 ml. of 0.6N hydrochloric acid and making the total volume to 1000 ml. in a volumetric flask. Five ml. of this solution were transferred by pipette to a 100 ml. volumetric flask and the solution made up to the mark with water. This final solution, containing 0.05 mg. of pigment in 100 ml. was compared in a one cm. cell in the spectrophotometer. Figure 1 shows the visible and ultraviolet absorption spectrum of the TBA pigment derived from rancid salmon oil.

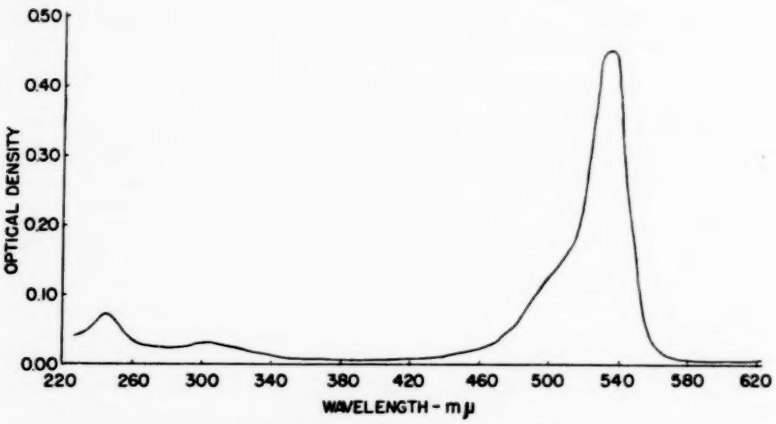

Figure 1. The absorption spectra of the crystalline TBA pigment isolated from rancid salmon oil.

The spectra obtained with the TEP and sulfadiazine pigments were identical but were not included in the figure for reasons of clarity. The main absorption maximum is at 532-535 mμ. with slight secondary maximas at 245 mμ and 305 mμ. TBA which has a primary absorption maxima in water at 264 mμ and an approximate $E^{1\%/1cm}$ of 1,170 does not appear to be present as a contaminant in the pigment samples. The $E^{1\%/1cm}$ values obtained for the 3 pigments, as prepared, gave the following initial values: malonaldehyde — 4610; sulfadiazine — 4580; and salmon oil — 4520. After purification and careful drying under vacuum, somewhat higher values were obtained. An average of all values obtained gave an $E^{1\%/1cm}$ of 4780.

Employing the value for the molecular extinction coefficient recently reported by Sinnhuber and Yu *(18)* of 1.56×10^5 and the $E^{1\%/1cm}$ value, the molecular weight of the pigment may be calculated. The calculated value obtained for molecular weight is 327 which agrees quite closely with the molecular weight of a compound with two molecules of TBA and one of malonaldehyde.

Molecular composition of the pigment. As was mentioned earlier in this paper, there are several theories as to the molecular relationship of TBA to malonaldehyde but there is no definite evidence. Since the determination of malonaldehyde is quantitative *(18)* and the pigment appears quite insoluble in dilute hydrochloric acid, experiments were conducted to determine yields with various combining ratios. Evidence so obtained could suggest an answer to the chemical composition of the pigment. Various molecular ratios of TEP to TBA were used: 1:1; 1:2; and 1:3. The yields, based on the TEP used, were respectively, 45%; 92%; 98%. According to the results obtained, the authors conclude that the pigment from TEP described in this report is a condensation of one molecule of malonaldehyde with two molecules of TBA. Although no definite evidence was obtained, the authors suggest the possible elimination of two molecules of water to complete the reaction.

The reaction between sulfadiazine and TBA is not easily explained. The yield data and combining ratio did not give any information about the chemistry of this reaction. The molecular extinction coefficient reported by Shepherd *(16)* was duplicated if the reaction conditions were met. However, increasing the TBA concentration was found to give a higher molecular extinction. The properties of the TBA sulfadiazine pigment were the same as the malonaldehyde pigment. However, the mechanism of the cleavage of the pyrimidine ring to yield a 3-carbon fragment which gives the same pigment as TEP remains to be explained.

Chemical composition of pigments. The purified crystalline pigments were subjected to quantitative microanalysis. Assuming a condensation of one molecule of malonaldehyde with 2 molecules of TBA and the elimination of 2 molecules of water, a molecular weight of 324.35 may be calculated. The assumed empirical formula would then be $C_{11}H_8N_4O_4S_2$. The analytical results obtained are shown in Table 1. The nitrogen

TABLE 1
Elemental Analysis of TBA Pigments

Element	Theory [1]	Malonaldehyde	Salmon Oil	Sulfadiazine
Carbon	40.76	39.62	38.64	38.54
Hydrogen	2.47	2.97	2.87	2.66
Nitrogen	17.28	15.75	15.78	15.34
Sulfur	19.75	19.06	18.51	18.54

[1] Assuming a molecular weight of 324.35 and formula of $C_{11}H_8N_4O_4S_2$.

determinations were by the Dumas method and sulfur by the Carius procedure. No ash was present after the carbon-hydrogen determination. From the data presented in this table, it appears that the TBA pigment prepared from malonaldehyde, salmon oil, and sulfadiazine are identical. The analytical results, however, suggest a slightly different empirical formula. It is suggested that one molecule of water of crystallization may be present. This more nearly corresponds to the elemental chemical analysis, but remains to be clearly demonstrated.

Paper chromatography of TBA reaction products. After numerous unsuccessful attempts to chromatograph the TBA red pigments, a solvent system was found which permitted paper chromatography of the red TBA reaction products. The system of Boulanger, 1951, described by Block, Le Strange and Zweig *(3)* gave a satisfactory rate of development. This system has the following composition: Phenol-isopropanol-formic acid-water, (80:10:10:100 v/v). The upper layer is used after allowing the mixture to stand for 24 hours. Whatman No. 1, filter paper, chromatographic grade, was used. Water solutions of the pigments or the pigments evolved in the Yu, Sinnhuber TBA procedure *(20)*, in the case of the 2 fishery products used, were applied to the paper by the usual technique. The chromatogram was developed descendingly for 8 hours at room temperature in a glass jar. Results obtained are shown in Figure 2. Inspection of the chromatogram with ultraviolet light revealed a fluorescent rapidly moving material which proved to be TBA. TBA fluorescent spots were noticed in the herring meal and tuna samples but not in the pigment preparations from TEP, sulfadiazine, or from rancid salmon oil. This procedure can serve as a means of separating pigment from TBA, which has been reported as a difficult procedure *(6)*. The Rf values of the pigments were as follows: Red pigment from sulfadiazine — 0.22; rancid salmon oil — 0.21; malonaldehyde (TEP acid hydrolysis

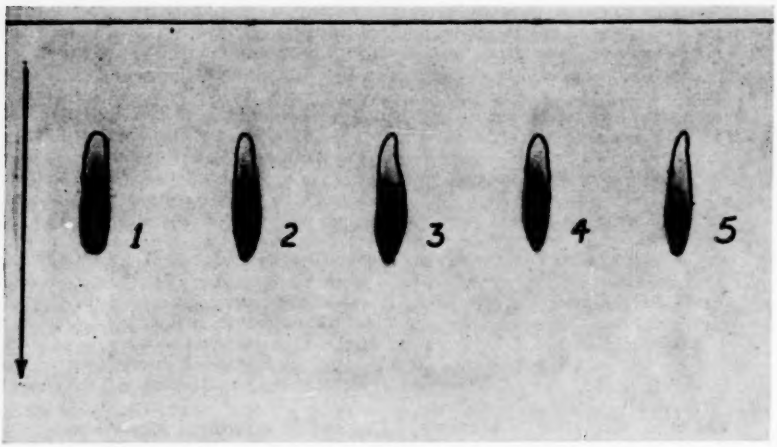

Figure 2. Chromatogram of TBA pigment prepared from various sources.

1. Sulfadiazine
2. Rancid salmon oil
3. Malonaldehyde from TEP
4. Frozen tuna
5. Herring meal

product) — 0.22; frozen tuna — 0.22; herring meal — 0.22. One may regard the chromatographic data as presumptive evidence that the pigments are identical.

Miscellaneous properties of the pigment. Figure 3 shows a photomicrograph of the TBA reaction product derived from rancid salmon oil. The crystals are purple black needles which gave no definite melting point up to 300° C. Identical crystals were obtained by the reaction of TBA with either sulfadiazine or TEP.

The pigment is only very slightly soluble in water, but is readily soluble in dilute

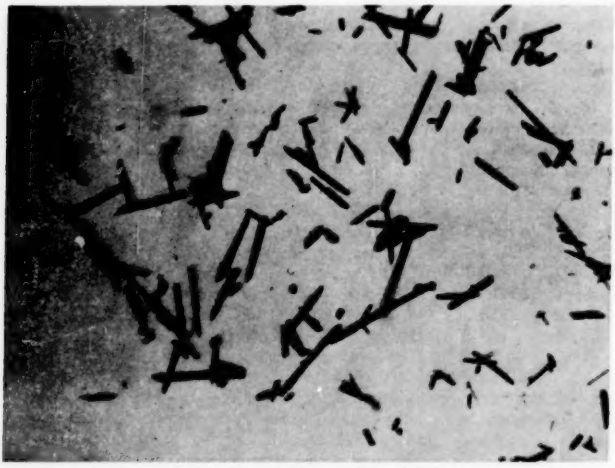

Figure 3. TBA pigment crystals prepared from rancid salmon oil, 120X.

alkaline or pyridine. The pigment may be precipitated from pyridine solutions by the addition of an excess of diethyl ether. The ether precipitated pigment was not crystalline, but an amorphous appearing compound difficult to filter and purify.

Alkaline solutions of the pigment gave a decrease in intensity of the spectral absorbence and shifted the maxima to a longer wave length. This point should be considered in TBA rancidity determinations made in alkaline or pyridine solutions. The pigment appears to be fairly stable in mild alkali. The wave length shift is reversible upon addition of acid.

The TBA malonaldehyde pigment is readily absorbed on cellulose powder or on filter paper pulp from mild acid solutions and may be eluted by weak alkali, phenol or pyridine solutions.

DISCUSSION

This paper presents evidence that the TBA reaction product of rancid salmon oil, sulfadiazine and malonaldehyde all yield the same pigment compound. The crystalline TBA derivatives were prepared and isolated from these three sources and appear to have identical properties. The molecular configuration of the pigment is believed to be a condensation product of two molecules of TBA with one molecule of malonaldehyde. The yield data and combining ratios support this contention. The elemental analytical results are additional evidence that the pigment is formed as a 2 to 1 mole reaction. The empirical formula derived is $C_{11}H_8N_4O_4S_2$, with a calculated molecular weight of 324.35. Molecular weight determinations were not attempted because of the slight solubility of the pigment.

Paper chromatography of the three pigments is offered as more proof that they are the same compound. The TBA pigment developed from herring meal and frozen tuna gave the same Rf values as the crystalline pigments. This technique also furnished evidence of the purity of the pigments.

Visible and ultraviolet spectrophotometry provided more data that the pigments were identical and was indicative of their purity and intense color. The average $E^{1\%/1cm.}$ value of the purified crystalline pigments was 4780 at 535mμ in water (pH — 0.9 with hydrochloric acid). The molecular extinction coefficient at 535mμ of the pigment derived from the acid hydrolysis product of TEP (malonaldehyde) was 1.56×10^5 (16). These values suggest that the pigment may be in a quinonoid configuration. The reaction and proposed structure of the TBA pigment are presented in Figure 4. The possibility of water of crystallization as part of the red pigment is suggested.

The data presented here show that the TBA reaction products of rancid salmon oil, sulfadiazine and malonaldehyde are identical. The oxidative origin of malonaldehyde in rancid fats or actual proof of this carbonyl as the free compound is still somewhat obscure. If one assumes that malonaldehyde is the compound necessary for the TBA test, then it is even less clear how sulfadiazine could give rise to malonaldehyde. In 1928, Powick *(14)* explained the mechanism of the Kreis test as due to the reaction between phloroglucinol and a 3-carbon aldehyde, epihydrin aldehyde. He suggested that the unstable aldehyde probably existed in fat as an acetal compound since he was unable to isolate the free compound. According to Lea *(10)* this view has found general acceptance. Lea adds "it is also possible that the aldehyde does not exist, even as an acetal, in the fat, but is formed from some precursor, possibly a peroxide, under the influence of the acid used in the (Kreis) test." Patton, Keeney, and Kurtz *(11)* investigated the Kreis

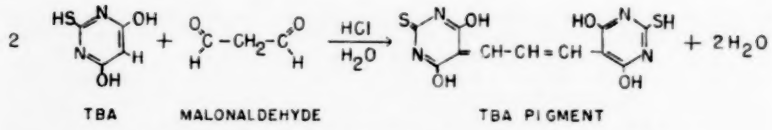

Figure 4. Proposed formation of TBA pigment from malonaldehyde.

reaction and concluded that malonaldehyde, not epihydrin aldehyde, was the responsible compound for both the Kreis and TBA test. They were unable to demonstrate the actual existence of either compound as the free aldehyde in oxidized fat. In 1958, Sinnhuber and Yu *(18)* used TEP as a standard for the TBA reaction. The identical color was formed from both malonaldehyde and from a substance in rancid fishery products. However, no proof was offered that malonaldehyde existed in rancid fat. It is possible that the TBA reagent on contact with rancid fat could hydrolyze an acetal derivative as suggested by Powick in the Kreis test or decompose a peroxide as offered by Lea. In this investigation a small amount (*2%*) of the total TBA reactive material was removed by a stream of nitrogen. It is suggested that free malonaldehyde was among the volatile material. The results are not conclusive since the amount was very small and a volatile malonaldehyde derivative or precursor may have passed over and then was hydrolyzed by the TBA reagent. The rancid oil had a TBA number of 287, expressed as mg. of malonaldehyde per 1000 g. of fat. This would mean that from 5 to 6 mg. of malonaldehyde were present as the free compound. Additional confirmation on this point is needed. When acid is added to the rancid fat, large quantities of volatile TBA reactive material were produced. Again the reasoning of either Powick or Lea would give an adequate explanation. It does show that a very volatile TBA reactive compound was split from the rancid fat by hydrochloric acid and indicates that the active TBA material is present as a larger molecule.

The reaction of sulfadiazine with TBA to yield the identical TBA malonaldehyde pigment is presented in this report. Kohn *(7)* first reported on the red color reaction between sulfadiazine and TBA. Shepherd *(16)* made a comprehensive study of the reaction between TBA and many pyrimidines. He concluded that the TBA red reaction was specific for the pyrimidine ring providing there were no substituents in the 4-, 5-, or 6-positions. The nature of the reaction was unknown. He reported a molecular extinction coefficient for the TBA reaction product of sulfadiazine of 6.5×10^4 at a pH of 2.1. We wish to report that, using the same procedure as Shepherd, the molecular extinction coefficient could be varied by changing the concentration of TBA. It appears that if suitable conditions were found, a molecular extinction coefficient identical with TEP is conceivable. The nature of the reaction whereby a 3-carbon fragment is apparently cleaved from a pyrimidine ring remains to be elucidated and extended to other compounds of similar structure. Unknown also are the other products of this reaction. This report confirms the absorption spectra of the red pigment reported by Shepherd *(16)* and the shift to a longer wave length, in an alkaline solution, with a decrease in absorbency. The chemical properties and reactions of the red pigment were extensively investigated by Shepherd. The results he obtained may be applied to the TBA malonaldehyde pigment.

SUMMARY

The pure crystalline TBA pigment was prepared as purple black needles from rancid salmon oil, sulfadiazine and the acid hydrolysis product of 1, 1, 3, 3-tetraethoxypropane, malonaldehyde. The results obtained by elemental analyses, absorption spectrophotometry, and paper chromatography all suggest the pigments are identical. The data indicate that the crystalline pigment is a condensation product of one molecule of malonaldehyde with two molecules of TBA and the probable elimination of two molecules of water. From these results and the data on yields and combining ratios, a structural formula is proposed and the empirical formula, $C_{11}H_8N_4O_4S_2$, or $C_{11}H_8N_4O_4S_2 \cdot H_2O$ is presented. Visible and ultraviolet spectrophotometry of the pigment confirm the primary maxima at 532-535 mμ with an average $E^{1\%/1cm.}$ of 4780 at a pH of 0.9. Secondary maxima are evident at 245 mμ and 305 mμ.

Most of the TBA reactive material is present in rancid salmon oil as complex compounds, possibly acetals, which are not volatile in a stream of nitrogen. However, upon the addition of hydrochloric acid, the TBA reactive material, probably malonaldehyde, may be removed by a stream of nitrogen. A small amount, 2 percent, appeared to be present as the free malonaldehyde.

Sulfadiazine reacts with TBA in a hydrochloric acid media to yield the identical pigment. The mechanism of the cleavage of the pyrimidine ring to yield a 3-carbon fragment remains to be explained.

Acknowledgment

The authors are very grateful to D. W. Glennie of the Forest Products Research Center, Corvallis, Oregon, for his helpful suggestions. We wish to extend our thanks to Kay-Fries Chemicals, Inc., of New York, New York, for the generous samples of 1, 1, 3, 3-tetraethoxypropane and 1, 1, 3, 3-tetramethoxypropane and to Union Carbide and Carbon Chemicals Company, New York, New York, for a sample of 1, 1, 3, 3-tetraethoxypropane.

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DETERMINATION OF CHLOROPHYLL AND PHEOPHYTIN IN BROCCOLI HEATED BY VARIOUS PROCEDURES*

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Measurement of the relative amounts of chlorophyll and pheophytin present in vegetables is of importance because it provides information regarding the condition of the vegetable when received. Changes occurring during the cooking process can also be followed by measuring progressive conversion of chlorophyll to pheophytin.

Estimation of chlorophyll in plant pigments depends fundamentally on the complete extraction of the pigment in known form from the plant material and on a reliable measurement of the pigment content of the extract. Pigments can be measured by colorimetry, fluorometry, or by estimation of magnesium content. Of these, only the colorimetric methods have been used for routine purposes.

Colorimetric procedures cover a wide range of complexity. A method described by Petering, Wolman and Hibbard *(7)* consists merely in extracting the plant material with 85% acetone and measuring the green color at the wavelength of maximum absorbance. Other more elaborate procedures, *(9), (10), (12)* depend upon separation of chlorophyll components by chromatography and determining each component by colorimetric measurement.

In a study carried out in our laboratories it was necessary to determine the percentage of chlorophyll converted to pheophytin in broccoli cooked by the various methods being investigated. A modification of the method of Petering *et al.*, because of its simplicity, was used in the initial phases of the work.

The absorbance of acetone extracts at 660 and 642.5 mμ was used for estimating chlorophyll content. Although pheophytin also absorbs at these wavelengths, its absorbance is considerably less than that of chlorophyll. Therefore, as chlorophyll is converted to pheophytin during the cooking process it is possible to follow the progress of the conversion by measuring the decrease in absorbance at these wavelengths.

The chlorophyll content of the broccoli was correlated with results obtained by panel evaluation of color; the panel evaluation in turn correlated with objective measurements of color obtained by use of the Gardner Color Difference Meter *(1)*. Results are summarized in Table 1. All panel color evaluations were made on a 5-point scale, a score of 5 denoting maximum greenness. Gardner a_L color readings were obtained using a Medium Green Color Standard L 50.5, a_L-25.6, b_L 6.4.

As shown in Table 1, the correlations of chlorophyll with panel color evaluations are lower than those between Gardner readings and panel color evaluations. Mackinney and Weast *(5)* have pointed out that changes in

* Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 28, 1958.

TABLE 1

Comparison of methods of color evaluation of cooked broccoli

Qualities correlated	Sample type	N [1]	Correlation coefficient
Gardner $\frac{a_L}{b_L}$ vs. Panel color	Heads	156	0.84
	Stems	156	0.87
Chlorophyll $\frac{mmg.}{100\ g.}$ vs. Panel color	Heads and Stems	156	0.52
		156	
Chlorophyll $\frac{mmg.}{100\ g.}$ vs. Gardner $\frac{a_L}{b_L}$	Heads and Stems	156	0.57

[1] Number of pairs correlated.

physical structure of plant walls during the cooking process will affect adversely the correlation between chlorophyll content and organoleptic color evaluation. Also, because of the lack of uniformity of the broccoli used in the study, there were large variations in its day-to-day chlorophyll content. It appeared, therefore, that the relative amounts of chlorophyll and pheophytin, rather than total chlorophyll, might correlate more closely with color evaluations obtained by other means.

A procedure for determining the percentages of chlorophyll and pheophytin in acetone extracts of frozen packs of peas and string beans has been reported by Mackinney and Weast *(5)*. It is based on the fact that chlorophyll can be quantitatively converted to pheophytin by treatment with oxalic acid. From results obtained by measuring the absorbance at 560 and 535 mμ, the relative percentages of the two substances were calculated.

A study was made of the Mackinney-Weast method in order to determine its suitability for measuring chlorophyll changes occurring in broccoli.

EXPERIMENTAL

Determination of absorption coefficients. The following formula has been developed by Comar and Zscheile *(2)* for calculating the amount of chlorophyll contained in ethyl ether extracts:

$$\text{Total chlorophyll (mg/l)} = 7.12 \log_{10} \frac{Io}{I} \text{ (at 660 m}\mu\text{)} + 16.8 \log_{10} \frac{Io}{I} \text{ (at 642.5 m}\mu\text{)}$$

To study the application of the Comar formula to acetone extracts it was necessary to compare the absorption spectra of chlorophyll and pheophytin in the 2 solvents. Broccoli was extracted with acetone and a portion of the extract transferred to ethyl ether, using the A.O.A.C. procedure *(6)*. A 25-ml. aliquot of the ether extract was treated with 1 ml. of a saturated solution of oxalic acid in ethyl ether. To a second aliquot was added 1 ml. of ethyl ether. Two 25-ml. aliquots of the acetone extract were treated respectively with 1 ml. of a saturated solution of oxalic acid in acetone and 1 ml. of acetone. The absorption spectra of these solutions was determined, using a Beckman DU spectrophotometer. From the data (Figure 1) thus obtained it was possible to calculate specific absorption coefficients for acetone extracts of chlorophyll, and for the change in absorbance occurring at various wavelengths when chlorophyll is converted to pheophytin. A comparison of the absorption spectra of chlorophyll in ether and in acetone at 642.5 and 660 mμ shows the following relationship:

$$\frac{\text{Absorbance (ether extract)}}{\text{Absorbance (acetone extract)}} = 1.22$$

Using these proportions, the Comar formula for calculation of total chlorophyll in ether extracts was modified for use in acetone extracts as follows:

$$\text{Total chlorophyll } \frac{\text{mmg.}}{\text{ml.}} = 8.69 \log_{10} \frac{Io}{I} \text{ (at 660 m}\mu\text{)} + 20.5 \log_{10} \frac{Io}{I} \text{ (at 642.5 m}\mu\text{)}$$

In the procedure of Mackinney and Weast *(5)* the percentage of chlorophyll and pheophytin in an extract of unknown composition is determined by measurement of its absorbance at 535 and 556 mμ. In the work on broccoli, determinations were based on measuring the change in absorbance rather than on absorbance as such. Aliquots of each extract were measured before and after treatment with oxalic acid. The chlorophyll present in the broccoli extracts was converted to pheophytin by treatment with oxalic acid and the change in absorbance utilized for determining the amount of chlorophyll originally present in the extract.

An examination of the absorption spectra, Figure 1, shows that at 558 mμ the absorbance of chlorophyll and pheophytin were about equal. Measurements at this wavelength were therefore used for determination of the total amount of chlorophyll and pheophytin present in the extracts. At wavelengths above 558 mμ the absorbance of chlorophyll was considerably greater than that of pheophytin. For this reason the possibility of utilizing measurements of absorbance changes at wavelengths other than 535 mμ was investigated. To do this it was necessary to determine specific absorption coefficients for changes in absorbance occurring at various wavelengths when chlorophyll is converted to pheophytin.

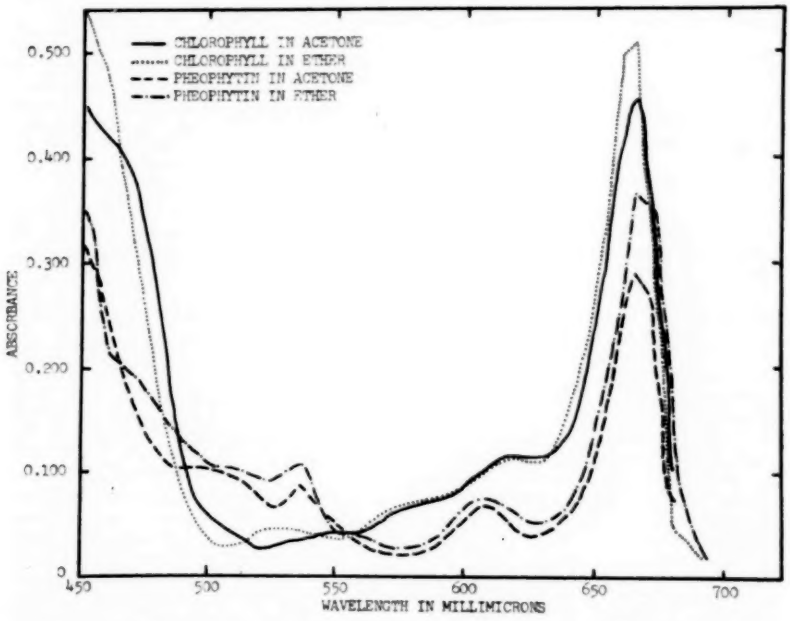

Figure 1. Comparison of the absorption spectra of chlorophyll and pheophytin in acetone and ether.

In Table 2 are shown constants obtained at various wavelengths for the absorption changes occurring when oxalic acid is added to extracts of uncooked broccoli. The specific absorption constants obtained at 558 mμ are also known.

TABLE 2

Effect of oxalic acid on absorbance of acetone solutions of chlorophyll

	Specific absorption coefficients at certain wavelengths			
	Increase 535 mμ	Decrease		Absorbance 558 mμ
		642.5 mμ	665 mμ	
Absorption coefficient	7.6	12.8	25.8	6.8
Standard deviation	0.232	0.339	1.34	0.316
Coefficient variation	3.1	2.6	5.2	4.6
Number of replications	14	14	14	14

Using these constants the percentage retention of chlorophyll in acetone extracts of broccoli was calculated from the following equations:

In extract of uncooked broccoli:

$$\text{Total chlorophyll} = 8.69 \log_{10} \frac{Io}{I} \text{ (at 660 m}\mu\text{)} + 20.5 \log_{10} \frac{Io}{I} \text{ (at 642.5 m}\mu\text{)}$$

In extract of cooked broccoli:

$$(1)\ \text{chlorophyll retained} \frac{\text{(mmg.)}}{\text{ml.}} = \frac{\text{change in absorbance}}{3} \left(\frac{\text{at 535 m}\mu}{.0076} + \frac{\text{at 642.5 m}\mu}{.0128} + \frac{\text{at 665 m}\mu}{.0258} \right)$$

$$(2)\ \text{Total chlorophyll + pheophytin} \frac{\text{(mmg.)}}{\text{ml.}} = \text{absorbance at} \frac{558\ \text{m}\mu}{.0068}$$

$$(3)\ \text{Percentage retention of chlorophyll} = \frac{(1)}{(2)} \times 100$$

In all cases the absorbance values are those obtained using a sample cell 1 cm. in width. The difference in molecular weight between chlorophyll and pheophytin was disregarded.

EFFECT OF OXALIC ACID ON CAROTENES AND XANTHOPHYLLS

In making the above calculations it is assumed that any changes in absorbance at the wavelengths of measurement are due entirely to the conversion of chlorophyll to pheophytin. Willstatter and Stoll *(11)* have stated that the yellow pigments present in plant extracts are sensitive to acids. Since the acetone extracts studied contained carotene and xanthophyll in addition to chlorophyll, it was necessary to investigate the effects of the oxalic acid treatment on the absorbance of these substances.

Extracts for this study were prepared by blending 100 g. broccoli with 100 ml. acetone. Twenty grams of the resulting slurry were extracted with 80 ml. acetone. The filtered acetone extract was placed in a separatory funnel and 20 ml. H_2O and 45 ml. petroleum ether were added. The H_2O acetone layer was discarded and the petroleum ether layer chromatographed on a 1-1 mixture of MgO and celite. The carotene was eluted with 10% acetone in petroleum ether, and xanthophylls with 20% acetone in petroleum ether. Eluates were diluted to 25 ml. and were treated with acetone and with oxalic acid as in the chlorophyll procedure. A comparison of the absorbance of the acetone and oxalic acid treated extracts is given in Table 3. Extracts used were approximately 5 times as concentrated as those used in determining chlorophyll.

As a result of this study it was concluded that any changes in absorbance caused by treating carotene or xanthophylls with oxalic acid, would have no significant effect on chlorophyll values as determined by the procedure described.

TABLE 3

Effect of oxalic acid on absorbance of carotene and xanthophylls

Wavelength, mμ	Absorbance of carotene		Absorbance of xanthophylls	
	In acetone	In acetone + oxalic acid	In acetone	In acetone + oxalic acid
450	0.072	0.072	0.462	0.459
535	0.0	0.0	0.017	0.015
558	0.012	0.012	0.0	0.0
642.5	0.0	0.0	0.0	0.0
660	0.0	0.0	0.0	0.0

RELATIVE STABILITY OF CHLOROPHYLL A AND CHLOROPHYLL B

In the method of Mackinney and Weast only the difference in absorbance at 535 mμ is used as a measure of the percentage of chlorophyll and pheophytin. At this wavelength the absorbance changes caused by conversion of chlorophyll to pheophytin were believed to be about equal for both chlorophyll a and chlorophyll b. At wavelengths above 535 mμ they were not equal. Joslyn and Mackinney *(4)* have reported that chlorophyll a and chlorophyll b, when treated with very dilute solutions of oxalic acid, are not converted to their respective pheophytins at the same rate. Any difference in the rate of conversion would affect chlorophyll values, particularly those calculated on the basis of changes in absorbance at wavelengths above 535 mμ. It was necessary, therefore, to study the behavior of chlorophyll a and chlorophyll b in broccoli cooked for various periods of time. As a preliminary step in this study the 2 forms of chlorophyll were separated using the procedure that follows.

Extraction. One-hundred grams of broccoli were blended for 2 minutes at medium speed with 100 ml. acetone. Thirty grams of the resulting slurry were blended for 5 minutes at reduced speed with 70 ml. acetone. Extract was filtered on sintered glass funnel, using suction and the residue washed with 50 ml. acetone. Residue was returned to the blender and blended for 2 minutes with 50 ml. acetone. It was then filtered through the same funnel and the combined filtrates diluted to 200 ml. with 80% acetone.

A 50 ml. aliquot of the extract was placed in a separatory funnel containing 50 ml. petroleum ether. Water was carefully added until all green pigments were transferred to the petroleum ether layer. The water-acetone layer was discarded and the last traces of acetone removed from the petroleum ether fraction by repeated bubbling through water, using the procedure described in A.O.A.C. *(6)*. The acetone-free fraction was then dried over anhydrous Na_2SO_4. All solutions used in the extraction and chromatographic procedures must be anhydrous and free of acid or alkali.

Chromatographic Procedure. *Preparation of column.* The absorbent consisted of powdered sucrose containing 3% corn starch. It was dried for 12 hours at 100° C. previous to using. The Allihn chromatographic tube 20 x 100 mm. was fitted into a bell glass by means of a one-hole rubber stopper, and the bell glass connected to a suction device, making use of pressure tubing. After starting the suction the tube was filled to within approximately 1½ inch of its top; absorbent was added in small portions and tamped into position after each addition: A ¼-inch layer of Na_2SO_4 was then added to the column.

Chromatography. After packing the tube, absorbent was prewashed with 25 ml. petroleum ether. Twenty milliliters of the chlorophyll extract, contained in petroleum ether, was then added to the column. The column was eluted with a mixture of 3 volumes benzene and 1 volume petroleum ether until all xanthophylls and carotene had been washed from the column. Eluate was collected in a small Erlenmeyer. Chlorophyll a was then eluted using 5% acetone by volume in petroleum ether. After removal of chlorophyll a, chlorophyll b was eluted using 10% acetone in petroleum ether. In the case of extracts of cooked broccoli it was also necessary to separate the pheophytins. These were eluted from the column in advance of their respective chlorophylls.

Elutions were carried out with the suction adjusted so that the flow of the eluate was approximately 100 drops per minute. Aliquots of the chromatographed solutions were converted to their corresponding pheophytins by treatment with oxalic acid. The absorption spectra, Figures 2 and 3, were determined using a Beckman DU spectrophotometer.

Absorption spectra of chlorophyll a, Figure 2, shows at 535 mμ a pheophytin peak and at 665 a sharp decrease in absorbance upon conversion of chlorophyll a to pheophytin. The corresponding spectra for chlorophyll b, Figure 3, also shows an increase in absorbance at 535 mμ when chlorophyll is converted to pheophytin. However, the maximum is at about 530 rather than 535 mμ and at no point is there a sharp peak similar to that of pheophytin a. The chlorophyll b has an absorption maximum at about 645 mμ and there is a sharp decrease in absorbance upon conversion to pheophytin.

Since the chromatographed extracts were in petroleum ether containing 10% acetone it was necessary to compare the absorbance of chlorophyll in this solvent mixture with that in ethyl ether. Measurements taken at 535, 568, 581, 589, 600, 642.5, 660 and 665 mμ indicated that:

$$\frac{\text{absorbance in ethyl ether}}{\text{absorbance in petroleum ether + acetone}} = 1.29$$

By application of this correction factor the Comar formula could be used for calculation of the chlorophyll content of petroleum ether-acetone extracts.

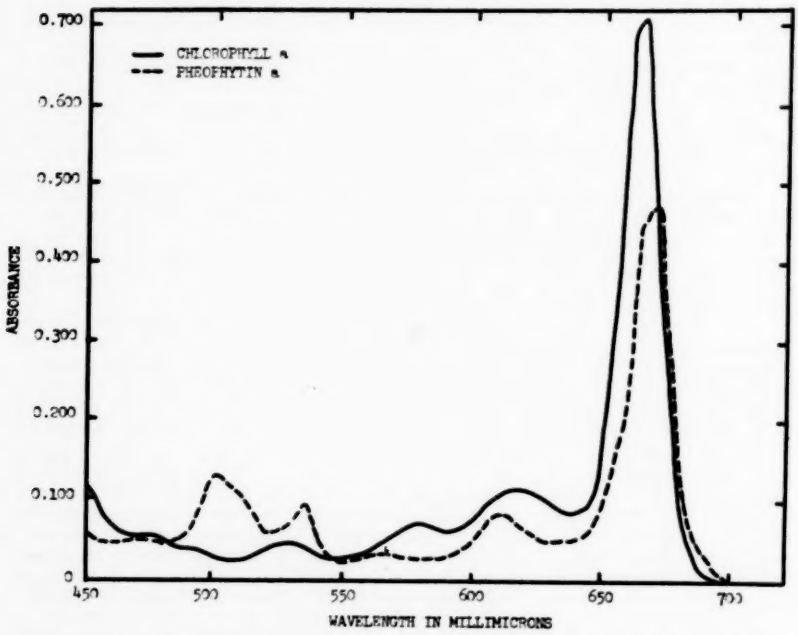

Figure 2. Absorption spectra of chlorophyll a and pheophytin a in petroleum ether containing 10% acetone.

RESULTS

Table 4 shows the percentages of chlorophyll retained in broccoli cooked for various times. These calculations were made on unchromatographed acetone extracts treated with oxalic acid in the usual manner. Also shown are the ratios of chlorophyll a to chlorophyll

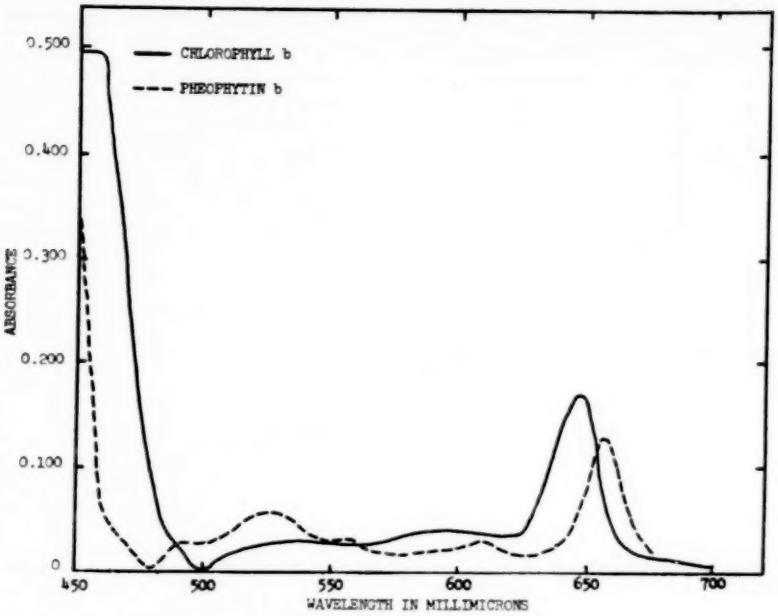

Figure 3. Absorption spectra of chlorophyll b and pheophytin b in petroleum ether containing 10% acetone.

TABLE 4

Percent retention of chlorophyll a and chlorophyll b in cooked broccoli

Cooking time minutes	Ratio $\frac{\text{chlorophyll a}}{\text{chlorophyll b}}$	% Chlorophyll retention		
		Total chlorophyll	Chlorophyll a	Chlorophyll b
0	2.03	—	—	—
5	1.77	82.5	78.7	90.3
10	1.06	58.9	45.2	86.7
15	1.00	47.0	35.1	71.2
20	0.65	31.3	18.4	57.6

b. These ratios were calculated on chromatographed extracts making use of the fact that at 600 mμ the specific absorption constants of the two forms of chlorophyll are the same. As shown in Table 4, chlorophyll a is converted to pheophytin at a much more rapid rate than is chlorophyll b.

Relative stability in relation to cooking time is also shown in Figure 4. It would appear that loss in green color which occurs when broccoli is cooked is due chiefly to the conversion of chlorophyll a to pheophytin a. Because of the color difference between chlorophyll a and b, the effect of this conversion on the appearance of broccoli is probably even greater than indicated in Figure 4. Chlorophyll a has an intense blue-green color and undergoes a pronounced change in appearance when converted to pheophytin. Chlorophyll b, on the other hand, is yellow-green, somewhat similar in appearance to pheophytin. The conversion of an equivalent amount of chlorophyll b therefore produces a much less striking change in appearance.

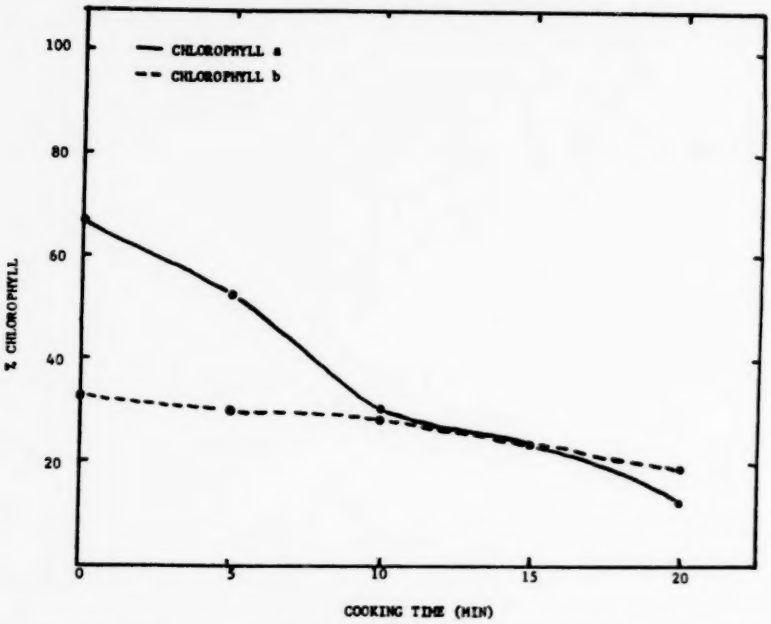

Figure 4. Relative stability of chlorophyll a and chlorophyll b in broccoli cooked for various times.

Table 5 shows percentages of chlorophyll retained in broccoli cooked for various times based on absorbance at 535, 642.5 and 665 mμ. In column 5 of the table are shown the averages of the values calculated at the 3 wavelengths. The percentage retention values of chlorophyll calculated at 642.5 were consistently higher than the corresponding values calculated at 535 mμ. This is due to the greater stability of chlorophyll b, since the change in absorbance at 642.5 mμ is due largely to the conversion of chlorophyll b to pheophytin. At 665 mμ, on the other hand, the change in absorbance is caused by the conversion of chlorophyll a to pheophytin; the low values obtained at this wavelength are a reflection of the greater instability of chlorophyll a.

Averages of the 3 values for the percentage retention of chlorophyll do not differ greatly from those calculated at 535 mμ alone. Measurements taken at 642.5 and 665 mμ serve as a useful check on those taken at 535. Also since the specific absorption coefficients are larger at 642.5 and 665 than at 535 mμ, an error in reading of the instrument at

TABLE 5

Percent retention of chlorophyll in broccoli calculated from absorption data at various wavelengths

Cooking time minutes	N [1]	Chlorophyll retention % at wavelength, mμ			
		535	642.5	665	Average
5	3	72.1	78.7	70.6	73.8
10	3	61.3	69.4	55.7	62.1
15	3	45.4	56.3	39.1	46.9
20	3	35.8	43.8	28.6	36.0

[1] Number of replications.

535 mμ would produce a proportionally greater error in the result than would a similar error in instrument reading at the higher wavelengths. Values obtained at 642.5 and 665 mμ also provide a useful index of the relative rate of conversion of the 2 chlorophylls to their corresponding pheophytins without the necessity for a chromatographic separation.

In Table 6 are shown panel scores, Gardner color values, and percentages of chlorophyll retained in fresh and frozen broccoli cooked in an electronic oven for various periods of time. Chlorophyll values were calculated from the changes in absorbance at 535, 642.5 and 665 mμ. A comparison of methods of color evaluation is shown in Table 7. As in the first series of experiments, Table 1, chlorophyll determinations were carried out on samples of broccoli prepared for chemical analysis. Gardner and panel color evaluations were carried out on broccoli from the same lot, which was prepared separately.

TABLE 6

Comparison of methods of color evaluation of cooked broccoli

Broccoli type	N [1]	Cooking time minutes	Chlorophyll retention %	Gardner a/L	Panel score
Fresh	3	3	93.8	13.7	5.0
	3	6	74.2	11.1	4.2
	3	9	56.0	9.9	3.1
	3	12	46.6	8.3	2.4
Frozen	4	6	92.1	14.4	4.9
	4	9	87.7	12.8	4.4
	4	12	67.2	10.8	3.7
	4	15	56.5	9.5	2.1

[1] Number of replications.

TABLE 7

Correlation of results obtained by color evaluation methods using modified chlorophyll procedure

Methods	Sample	Fresh broccoli		Frozen broccoli	
		N [1]	r [2]	N [1]	r [2]
Chlorophyll vs. Panel	Heads and Stems	16	0.84	16	0.85
Chlorophyll vs. Gardner	Heads and Stems	12	0.84	16	0.73
Gardner vs. Panel	Heads	12	0.93	16	0.86
	Stems	12	0.83	16	0.81

[1] Number of paired samples.
[2] Correlation coefficient.

Since the factors being correlated were not determined on identical samples, it was not possible to determine the extent to which they were influenced by sample variation. All correlations, however, were highly significant. Those involving chlorophyll were considerably higher than those obtained in the first series of experiments, Table 1, in which chlorophyll values were based merely on measurement of the green color at its wavelengths of maximum absorbance. It would appear that as a means of color evaluation chlorophyll retention as determined by the method described here, compares favorably in precision with results obtained by Gardner or panel color evaluation. Chlorophyll determinations have an additional value since they provide a means of evaluating color directly in terms of that originally present.

EFFECT OF ZINC CHLORIDE ON COLOR OF ACETONE EXTRACTS

Willstatter and Stoll *(11)* have found that metals may be introduced into chlorophyll derivatives that have been formed by elimination of magnesium, Figure 5. They were able to form complexes between chlorophyll derivatives and copper, zinc and iron, contained in solutions of glacial acetic acid or in alcohol.

It was believed that this property of chlorophyll might be of value in qualitative determinations. In order to investigate this possibility, samples of raw broccoli and of broccoli cooked by various methods were extracted with acetone. Aliquots of these extracts were treated with 2 ml. portions of acetone saturated with zinc chloride. After standing for one hour the extracts were filtered. The zinc chloride treatment appeared to restore much of the green color which had been dispelled by the cooking procedure. Spectrophotometric studies were made of untreated acetone extracts of the broccoli and of comparable extracts treated with oxalic acid or with zinc chloride.

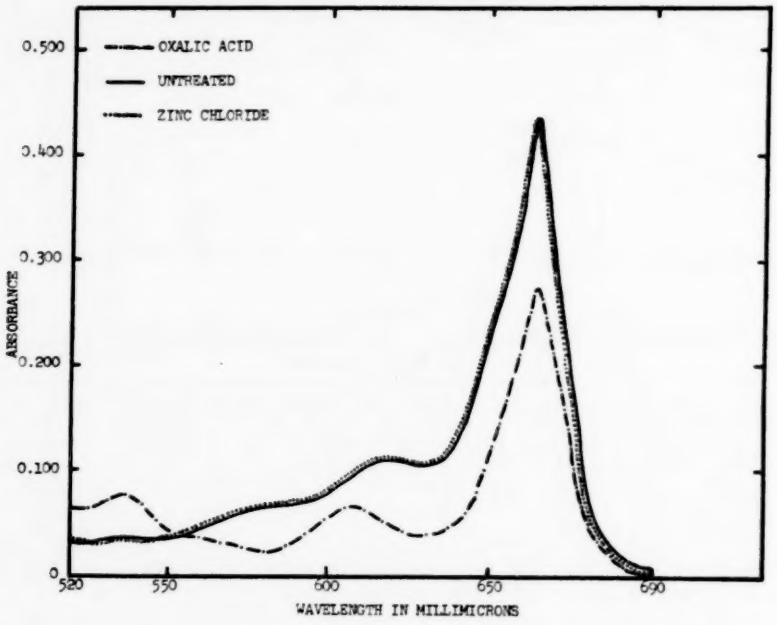

Figure 5. Absorption spectra of acetone extract of raw frozen broccoli

Figure 5 shows results obtained using extracts of raw broccoli. The extract of uncooked broccoli contained very little pheophytin and consequently treatment with zinc chloride produced no marked change in the absorption spectra. Treatment with oxalic acid, on the other hand, converted the chlorophyll to pheophytin, causing significant changes in absorbance.

Figure 6 shows the spectra of extracts of broccoli which had been cooked for 1 minute at 15 pounds pressure. Since the most significant changes occur at wavelengths between 535 and 665 mμ only that portion of the spectra is shown. The untreated extract had an absorption spectra approximately midway between that of the zinc chloride and the oxalic acid-treated extracts indicating that in this sample there had been a partial conversion of chlorophyll to pheophytin. Treatment with zinc chloride caused a restoration of green color as indicated by the increase in absorbance at wavelengths above 558 mμ. The zinc chloride-treated extract had a spectra similar to, but not identical with that

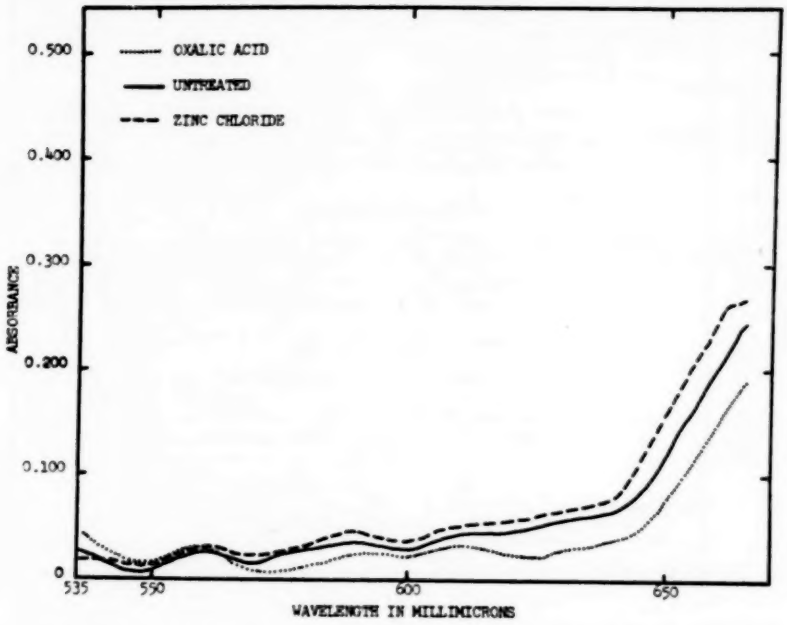

Figure 6. Absorption spectra of acetone extract of broccoli cooked 1 minute at 15 pounds pressure.

of chlorophyll. Treamtent with oxalic acid converted the unchanged chlorophyll to pheophytin, causing a decrease in absorbance at wavelengths above 558 mμ.

In Figure 7 is shown a portion of the spectra of extracts of broccoli cooked for a period of 5 minutes at 15 pounds pressure. In this case treatment with oxalic acid produced very little change indicating that most of the chlorophyll had already been degraded by the cooking process. Treatment with zinc chloride, on the other hand, produced a marked change in the absorption spectra. The absorbance of the zinc chloride treated extract, however, in contrast to that of other similarly treated extracts, Figures 5 and 6, is lower at 665 mμ than at 660 mμ. This may indicate that in the case of broccoli subjected to drastic cooking procedures, the chlorophyll is at least partially degraded beyond the pheophytin stage.

Absorption constants for the change-in-spectra produced by addition of zinc chloride to extracts containing mixtures of chlorophyll and pheophytin were quite variable. With further work it might be possible to develop reproducible constants. Use of zinc chloride, however, as described above, furnishes a useful qualitative check on the reliability of results obtained by the oxalic acid procedure.

DISCUSSION

Some of the factors influencing absorption spectra of chlorophyll are pH, the polarity of the solvent used *(3)* and the relative amounts of the various chlorophylls present in the extract. Although chlorophyll is believed to occur in plants, chiefly as chlorophyll a and b, small amounts may be present as c, d, and e, protochlorophyll, bacteriochlorophyll, and bacteriovirden *(9)*. Some of these have been characterized only by a name and a qualitative ab-

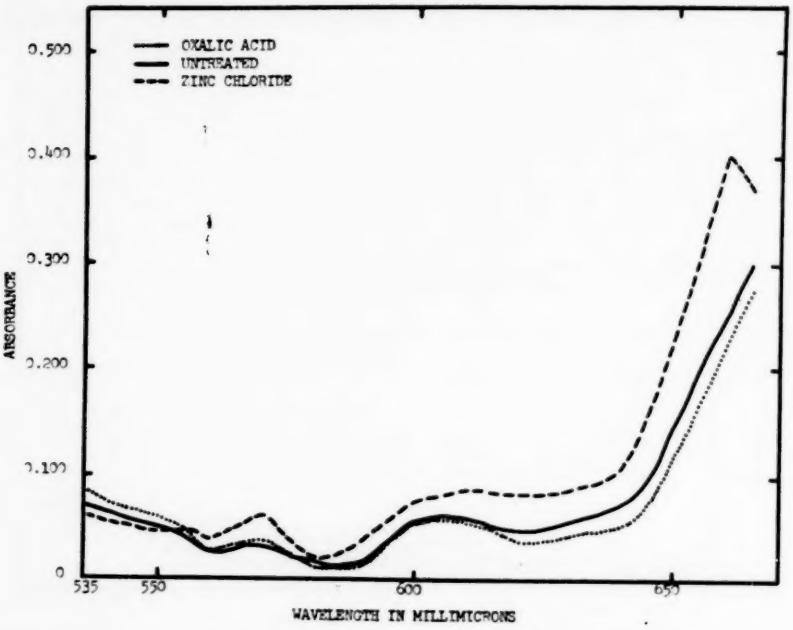

Figure 7. Absorption spectra of acetone extract of broccoli cooked 5 minutes at 15 pounds pressure.

sorption spectrum. Therefore, the analyst has no assurance of the uniformity of the isomeric composition of the chlorophyll extracts being investigated.

As pointed out by Smith and Benitez *(9)*, it would seem advisable for each analyst to develop his own analytical constants under the conditions of analysis used. The techniques described here should prove useful in the development of such constants.

SUMMARY

A method for determination of the relative amounts of chlorophyll and pheophytin present in extracts has been described. In the adaptation of this method to the study of the effect of cooking broccoli on its chlorophyll content, the following steps were taken:

1. The Comar formula for calculation of the total chlorophyll content of ether extracts was modified for use with acetone extracts.
2. Each extract was examined spectrophotometrically before and after complete conversion of the remaining chlorophyll to pheophytin by treatment with oxalic acid.
3. Absorption constants for the change in absorbance were determined at 535, 642.5 and 665 mμ, and from these constants the relative amount of chlorophyll present in the extracts was determined.
4. The effect of oxalic acid on the carotene and xanthophylls present in the extracts was determined.

5. Chlorophyll a and chlorophyll b were separated by chromatography and the relative stability of the two forms of chlorophyll in cooked broccoli was determined.

6. The chlorophyll values were correlated with color evaluations by palatability panel and by Gardner Color Difference Meter.

7. The possibility of using zinc to replace magnesium, eliminated from chlorophyll during the cooking process, shows promise.

Acknowledgments

Thanks are extended to Dr. Edward Toepfer for his helpful suggestions, to Miss Gladys Gilpin and Mrs. Velma Chapman who cooperated in the preparation of the broccoli and the evaluation of color by panel and Gardner Color Difference Meter, and to Mr. Jacob Eisen for assistance in statistical evaluation of the data.

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EFFECT OF FERTILIZERS ON QUALITY AND COMPOSITION OF TOMATO JUICE[a]

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(Manuscript received May 31, 1958)

The effect of fertilizer application on the metabolism *(6, 22)*, composition *(7, 9, 11, 19, 20)*, and yield *(8, 10, 12, 13, 18)* of tomato plants has been investigated. However, it has not been shown whether growing tomatoes under conditions favoring high yields would cause differences in the flavor and composition of the canned tomato juice.

In the present work, tomatoes of the Improved Pearson variety were grown under the same field conditions but with different treatments of nitrogen, phosphorus, and potassium fertilizers. The fruit was harvested under commercial field conditions and canned as juice in the pilot plant at Davis. Results on the effect of various fertilizer applications on some chemical constituents and on the flavor of canned tomato juice are presented.

EXPERIMENTAL MATERIALS AND METHODS

Tomato fruits. Tomatoes of the Improved Pearson variety were grown under various levels of fertilizer application on Livermore clay-loam soil in a commercial field in Alameda County, California. The field had been used for growing alfalfa the previous four years and no fertilizers had been applied. The tomato plants were transplanted in the field on May 11, 1956. Each plot consisted of 4 rows 93 feet long. Plants were set 18 inches apart; the distance between rows was 5 feet. From May 11-16, nitrogen, phosphorus, and potassium were applied 6 inches from the plants and 6 inches deep in various combinations and amounts. Fertilizer treatments are described in Table 1.

Tomatoes were harvested at canning maturity on September 19, 1956. Care was taken to obtain representative samples of uniform canning maturity as judged visually by skin color. Four replications were made for each treatment.

Canning of tomato juice. Each sample consisted of 120 lbs. of tomatoes. All the processing equipment was made of stainless steel to reduce Vitamin C loss. Fruit was washed with sprays of cold water, sorted, trimmed, and stemmed. It was then crushed in a "Spike-pump" with simultaneous steam heating to 185° F. The macerate was pumped into a paddle finisher with a 0.033-inch mesh screen. The extracted juice was pumped into a centrifugal vacuum deaerator under 20 inches of vacuum, and through a tubular heat exchanger for 15 seconds to a temperature of 198° F. The hot juice was pumped into a surge tank, where 0.75% of sodium chloride was added, and then filled into No. 2½ cans at 160° F. Cans were sealed under atmospheric pressure, processed in a rotary cooker at 210-212° F. for 30 minutes, and rapidly cooled in a rotary cooler to 100° F. Samples were stored at 68° F. for three months before chemical analyses and organoleptic evaluations were made.

Nitrogen. The standard Kjeldahl method *(3, 20)* was used for total nitrogen, the ninhydrin colorimetric method of Troll and Cannan *(21)* for amino nitrogen *(16)*, and the magnesium oxide distillation method *(3)* for ammonium nitrogen determination.

Soluble solids. Soluble solids content of tomato juice was determined with a Zeiss Opton refractometer.

pH. The pH was determined with a Beckman glass electrode pH meter.

Total titratable acidity. Ten ml. portions of the sample in 200 ml. of distilled water

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were titrated with 0.1 N NaOH to a pH value of 8.0, using a Beckman pH meter. Results were expressed in grams of citric acid per 100 ml. of tomato juice.

Ascorbic acid. The official 2, 6-dichlorophenol-indophenol titration method, as modified by the National Canners Association *(3, 17)*, was used.

Ash. The ash content of tomato juice was determined according to the official method *(3)*. The ash was used for subsequent determination of the salts.

Sodium chloride. The volumetric silver-nitrate-chromate method was used for the canned tomato juice *(17)*.

Phosphorus. Ashed samples were dissolved in 100 ml. of 1:4 HCl in the crucibles, and evaporated to dryness on a steam bath to precipitate the silica. The residue was dissolved in 20 ml. of 0.2 N HCl, filtered, and made up to 100 ml. with distilled water. This solution was used for the determination of P, K, Na, and Ca. The phosphates were determined colorimetrically by the ammonium molybdate method *(2)*. The blue color that developed was measured in a Klett-Summerson photoelectric colorimeter, using a red filter (No. 66).

Potassium, sodium, and calcium. The flame spectrophotometric method as modified for the determination of these elements in the plant tissue *(4, 5)* was used.

Flavor evaluation. The flavor of tomato juice was evaluated organoleptically by a panel of 7 persons who ranked 3 samples at a time according to the following scale: 1. *best*; 2. *intermediate*; and 3. *poor*. Four replications of the same fertilizer treatment were studied in each case, and 28 judgments were made for each sample. The experiment was designed to study the following variables:

(1) *Effect of the amount of nitrogen fertilizer.* The control sample was compared with two samples made from tomatoes fertilized with 60 and with 120 lbs. of ammonia nitrogen per acre (samples 5 and 6).

(2) *Effect of phosphates and potassium.* The control was compared with samples of juice from plots fertilized with N and P_2O_5 (120 lbs. per acre, sample 7) and N, P_2O_5 and K_2O (120 lbs. per acre, sample 4).

Analysis of variance. Data were analyzed statistically, using the Least Significant Difference (L.S.D.) as described by Alder and Roessler *(1)*. Flavor data were evaluated for significance, using the critical ratio method described by Krum *(14)*.

RESULTS

Nitrogen contents of tomato juice. The effect of field application of inorganic fertilizer on the nitrogen content of tomato juice is shown in Table 1. The average value of 4 replications for each treatment is used. It is evident that the samples of juice prepared from fertilized tomatoes contained slightly higher amounts of total, amino, and ammonium nitrogen. The inorganic nitrogen consists mainly of ammonium nitrogen, which ranged from 6.27 to 8.05% of the total nitrogen. Neither nitrate nor nitrite nitrogen was detectable in the samples of tomato juice examined. This was evidenced by the fact that reduction with salicylic acid and potassium thiosulfate *(3)* during digestion did not cause any increase in total nitrogen content as determined by the standard Kjeldahl method.

Amino nitrogen of the samples varied from 35.2 to 39.0% of the total nitrogen. All the samples from fertilized plants had a higher amino nitrogen content than did the control. The result is significant at the 5% probability level. A comparison of samples 1, 5, and 6 indicates that both yield and nitrogen content increased as the amount of ammonia fertilizer increased. A comparison of samples 1, 2, and 6 indicates that under the conditions of this experiment, ammonium and nitrate nitrogen were equally effective in increasing yield and in influencing nitrogen content of the fruit provided equivalent concentrations of nitrogen were used. When a complete fertilizer was used (sample 4, with 120 lbs. each of N, P_2O_5, and K_2O per acre), the concentration of amino nitrogen was not as high as in the samples from plots that were treated only with anhydrous ammonia (samples 5 and 6).

Soluble solids, acidity, and ascorbic acid. In Table 2, the effect of field fertilizer application on soluble solids, pH, total acidity, and ascorbic acid content of the canned tomato juice is presented. No significant difference in soluble solids content and pH was observed between the fertilized and unfertilized samples. The total acidity of the samples from fertilized plants was slightly higher than that of the control sample. Potassium fertilizer (samples 3 and 4) seemed to have little effect on the acidity of the juice.

TABLE 1

Effect of field fertilizer application on yield and nitrogen content of tomato juice
Alameda county, 1956 pack

Sample	Treatment	Nutrients added lbs./acre			Total yield tons/acre	Total N mg./100 ml.	Amino N		Ammonium N	
		N	P_2O_5	K_2O			mg./100 ml.	% of T.N.	mg./100 ml.	% of T.N.
1	control	0	0	0	22.7 ± 3.1	96.43	35.12	36.4	6.92	7.18
2	NH_4NO_3	120	0	0	29.2 ± 4.5	108.30	41.13	37.9	7.15	6.60
3	NH_4NO_3 TSP [1]	120	120	0	33.7 ± 3.3	107.90	42.03	39.0	7.18	6.65
4	NH_4NO_3 TSP [1] K_2SO_4	120	120	120	33.3 ± 4.2	109.40	38.48	35.2	6.85	6.27
5	NH_3	60	0	0	22.8 ± 6.4	104.50	40.82	39.0	7.34	7.02
6	NH_3	120	0	0	27.3 ± 4.6	107.40	41.95	39.0	8.10	7.55
7	NH_3 TSP [1]	120	120	0	33.3 ± 3.8	105.00	40.93	39.0	8.48	8.05
8	NH_3 TSP [1]	60	60	0	26.9 ± 4.0	103.60	39.35	38.0	7.74	7.47
L.S.D. at 5% probability level					2.55	3.27	1.96	—	1.25	—

[1] TSP = triple superphosphate ($CaHPO_4$)

TABLE 2

Effect of field fertilizer application on brix, acidity, and ascorbic acid content of canned tomato juice. Alameda county, 1956 pack

Sample	Treatment	Nutrients added			Soluble solids Brix at 20° C.	pH	Total acidity as % citric	Ascorbic acid mg./100 ml.
		N	P_2O_5	K_2O				
1	control	0	0	0	5.69 ± 0.22	4.40	0.315	18.0 ± 3.0
2	NH_4NO_3	120	0	0	5.61 ± 0.25	4.39	0.342	16.8 ± 3.6
3	NH_4NO_3 TSP	120	120	0	5.57 ± 0.28	4.44	0.336	16.5 ± 4.0
4	NH_4NO_3 TSP K_2SO_4	120	120	120	5.60 ± 0.18	4.43	0.341	17.2 ± 2.5
5	NH_3	60	0	0	5.67 ± 0.14	4.39	0.351	14.5 ± 1.1
6	NH_3	120	0	0	5.64 ± 0.13	4.40	0.362	15.3 ± 1.2
7	NH_3 TSP	120	120	0	5.47 ± 0.28	4.41	0.355	15.2 ± 2.8
8	NH_3 TSP	60	60	0	5.57 ± 0.17	4.34	0.364	14.3 ± 0.5

The ascorbic acid content of the juice from fertilized plants was slightly lower than that of the control sample and distinctly so in the cases where anhydrous ammonia was used (samples 5 to 8). It should be noted, however, that the ascorbic acid content of tomatoes is influenced more by the climatic conditions of the growing area than by the fertilizer application.

Inorganic constituents. Principal inorganic constituents of the various tomato juice samples are shown in Table 3. In general, no appreciable change in the inorganic constituents was observed.

Potassium is an important inorganic cation present in tomato juice. There seemed to be no important difference in potassium content between juices made from fertilized and those from control plants. However, a small increase in calcium and phosphorus content was observed in the samples from fertilized plots.

Color. Results are not presented because no significant differences in color were observed among the samples.

Flavor. The flavor of tomato juice was affected to a certain degree when fertilizers were used. Results are shown in Table 4. Highly fertilized tomatoes gave a less acceptable juice. The control sample was generally preferred, and the samples from fertilized fruit were described as having a more "acid" flavor. Flavor acceptance of samples from moderately fertilized fruit was practically the same as those from the untreated samples.

The effect of high ammonia nitrogen on flavor can be seen by comparing samples 1, 5, and 6. The sample with the highest amount of nitrogen (sample 6, 120 lbs. ammonia nitrogen per acre) was liked the least by the panel.

The effect of nitrogen, phosphorus, and potassium fertilizer on the flavor of the canned juice can be seen by comparing samples 1, 4, and 7. The control sample was preferred. Application of nitrogen, phosphorus, and potassium seems to cause an inferior flavor in the product. However, it is possible that the poor flavor might be caused by the effect of high nitrogen, as shown above. If so, then high potassium application (sample 4 as compared with samples 1 and 7) may or may not be a contributing factor. Over-fertilization could evidently have some undesirable effect on the quality of the juice. Proper control of the amount of plant nutrients for higher yield and good quality is essential.

TABLE 3

Effect of field fertilizer treatment on the inorganic constituents of canned tomato juice. Alameda county, 1956 pack

Sample	Treatment	Nutrients added lbs./acre			% Ash	% NaCl	K mg./100 ml.	Ca [1] mg./100 ml.	P [2] mg./100 ml.
		N	P_2O_5	K_2O					
1	control	0	0	0	1.18	0.868	215	4.9	9.9
2	NH_4NO_3	120	0	0	1.15	0.834	213	5.8	12.7
3	NH_4NO_3 TSP	120	120	0	1.15	0.830	225	6.0	13.4
4	NH_4NO_3 TSP K_2SO_4	120	120	120	1.16	0.862	221	6.6	12.9
5	NH_3	60	0	0	1.13	0.835	228	4.7	14.3
6	NH_3	120	0	0	1.18	0.857	231	5.3	13.0
7	NH_3 TSP	120	120	0	1.16	0.873	237	5.6	12.2
8	NH_3 TSP	60	60	0	1.13	0.857	217	5.9	11.1

[1] L.S.D. at 5% level = 0.81
[2] L.S.D. at 5% level = 2.72

DISCUSSION

Yield data shown in Table 1 indicate that the Livermore clay-loam soil used in this investigation responded equally well to NH_3 or NH_4NO_3 at the level of 120 lbs. N per acre. A slight increase in yield resulted from the application of triple superphosphate as shown in Table 1 (compare sample 2 with 3, and 6 with 7). Comparison of samples 3 and 4 shows that potassium did not cause any increase in yield.

Breon and Gillam *(6)* reported that tomato plants growing in a medium with nitrate as a source of nitrogen showed an accumulation of nitrates in all parts of the plant following rapid absorption rather than non-utilization. However, analysis of the tomato juice used in the present investigation did not show any detectable amount of nitrate when the official method of analysis *(3)* was used.

Lee and Sayre *(15)* have indicated that the use of potash fertilizer tends to increase the total acidity in the tomato fruits. In the present investigation, samples from fertilized plots showed slightly more acidity than the control but no particular increase in potassium ions. This discrepancy may have been caused by the difference in climate and soil conditions.

Application of the various fertilizers resulted in increases in the total, amino, and ammonium nitrogen in the canned tomato juice. The amino and ammonium nitrogen constituted a reasonably constant percentage of total nitrogen. Changes in flavor is more likely to be caused by dilution of flavor constituents because of higher yield or by the introduction of some unknown poor flavor constituents than by an increase in amino acids.

Hester *(11)*, reporting on fundamental factors influencing the composition of tomato puree, found samples from fertilized treatments to have a slightly higher acidity and nitrogen content and a lower ascorbic acid content than the control. His findings agree with the data collected here. The absolute quantity of ascorbic acid found in the present investigation was lower than that reported by Hester, a result that might be attributed to the differences in climatic conditions, yield per acre, processing or other conditions. For potassium-deficient soils, Hester reported that the application of potassium in mixed fertilizers improved the red color and increased the total solids, ash, sugar, Vitamin C, titratable acidity, and potassium content. Color in tomato fruits is known to be related to the lycopene and carotene contents which vary with the temperature to which the plants have been exposed, as well as with light intensity, abundance of leaf surface, and harvest maturity. An increase in color due to potassium fertilization was not found in the present investigation, possibly because there was no deficiency of this element in these field plots.

Tomato juice flavor is an important quality factor. Results shown in Table 4 indicate that the application of a high level of nitrogen (120 lbs. of ammonia nitrogen per acre) resulted in a juice less acceptable to the panel than that which came from an untreated plot. When a complete fertilizer was applied, as was the case with sample 4, the flavor of the resulting juice was less pleasing than that of the control. The increase in phosphates in the fruit, and the dilution of flavoring substances caused by the high yield, may have contributed in part to the change. The flavor constituents of tomatoes should be investigated further.

TABLE 4

Flavor evaluation of canned tomato juice with different fertilizer treatment. Alameda county, 1956

Sample	Fertilizer treatment	Flavor rank [1]	
1	control (no fertilizer)	1.64	
			NS
5	NH_3 (60 lbs. N/Acre)	1.75	*
			*
6	NH_3 (120 lbs. N/Acre)	2.60	
1	control (no fertilizer)	1.40	
			*
4	NH_4NO_3, TSP, K_2SO_4 (120 lbs./acre)	2.81	*
			*
7	NH_3, TSP (120 lbs./acre)	1.90	

NS = Not Significant
* = Significant difference at the 5% level.
[1] The smaller number represents a better flavor rating.

In conclusion, application of fertilizer in tomato production is to be encouraged because of the increase in yield and the reduction of production costs, although over-fertilization is to be avoided. Because of the variation in soil properties and climatic conditions from one field to another, it is difficult to formulate a fertilizer program that is applicable to all tomato fields. Results of this investigation suggest that variations in the quality and composition of tomato juice may be traced back to the cultural practices. Conditions that favor the production of a high-quality final product should be the goal of both producers and processors.

SUMMARY

The influence of various fertilizers applied to commercially grown tomatoes on the quality and composition of canned tomato juice was studied. Samples from fertilized plants were somewhat higher in total acidity, and samples from plants treated with nitrogen were higher in nitrogenous compounds. Mineral composition of the juice was not affected appreciably by the fertilizer application, although phosphorus content was slightly higher in the juice from fertilized fruit. Tomato juice flavor was affected to a certain degree when fertilizers were used. The juice of highly fertilized tomatoes was less acceptable, as evaluated by the ranking test. The control sample was generally preferred, and the samples from fertilized plants were described as having a more "acid" flavor. Flavor rating of the juice from moderately fertilized plants was practically the same as for the untreated samples.

Acknowledgment

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TOXIN PRODUCTION AND ORGANOLEPTIC BREAKDOWN IN VACUUM PACKAGED FRESH MEATS INOCULATED WITH *CLOSTRIDIUM BOTULINUM* *

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Vacuum packaging of non-processed perishable foods is now a widespread practice. Although these products are labeled "Keep Under Refrigeration", the possibility that they might be mishandled by the retailer and consumer is ever present. Vacuum packaged fresh meat would seem to present an excellent environment for the growth of *Clostridium botulinum*. Thus, the production of toxin in organoleptically acceptable product would constitute a serious hazard. On the other hand, if it can be demonstrated that toxin formation does not precede the point at which meat becomes obviously putrid, the element of danger is greatly diminished.

Segalove and Dack *(5)*, studying the formation of toxin in rehydrated dried beef, pork and ham inoculated with *Cl. botulinum*, observed obvious putrefaction in all of their toxic samples and no abnormal odor in toxin-free samples. Several observations of toxicity without overt organoleptic breakdown have been reported in the literature *(2, 3, 7, 9)*. However, none of these dealt with unprocessed meat.

This paper describes a series of experiments in which rates of toxin formation and putrefactive breakdown were studied in fresh meats seeded with *Cl. botulinum* spores and packed under anaerobic conditions.

MATERIALS AND METHODS

Spore inoculum. A water suspension was used, containing 1,000,000 spores per ml., consisting of 100,000 heat shocked spores of each of 5 Type A (62, 73, 78, 12885, 13824) and five Type B (32, 113, 213, 12033, 13983) strains of *Cl. botulinum*. This suspension was prepared by the Western Laboratory of the National Canners Association and supplied to one of us (Schmidt) for use in another study.

Meat variables. Comminuted fresh beef and pork were utilized as test materials in 3 different experimental systems:

1. Untreated
2. Ethylene Oxide Treated: The meat was placed in a vacuum jar which was then evacuated to 29" of vacuum. Ethylene oxide gas was introduced into the jar until 8" of vacuum remained. The apparatus was held 18 hours at 32-36° F., after which all hose clamps were opened to admit air into the system.
3. Irradiated: The test product was packed into saran bags which were then partially evacuated, heat sealed, flattened to 1/8 in. thickness and frozen at 0° F. Radiation was applied by means of a resonance transformer. Each package received a total exposure of approximately 3 megarep.

Inoculation and packaging. Approximately 10 g. of test materials were packed in thermal death time cans taking care to exclude air pockets. The meat was then

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inoculated with 0.1 ml. of the *Cl. botulinum* spore suspension using a 1 ml. tuberculin syringe and 26 gauge needle. Thus, the concentration in the product was 100,000 spores per can or 10,000 per gram of meat. The cans were closed with undercover gassing with nitrogen gas as described by Schmidt, Bock and Moberg *(4)*. This procedure has been shown to remove headspace oxygen equivalent to that obtained by 29.25 inches vacuum.

Incubation temperatures. 38°, 70°, 85°, and 99° F.

Organoleptic evaluation. Sample cans of each variable were removed from incubation at designated times, chilled and opened aseptically. The contents of each can were allowed to thaw at room temperature and shaken with 50 ml. of sterile water after examination for off-odor and appearance.

Filtrate production. Approximately 5 ml. of a meat-water suspension were placed in a sterile 13 × 100 mm. tube. Particulate material was separated from the water phase by pushing a sterile absorbent cotten plug through the suspension to the bottom of the tube. The resultant filtrate was used for intraperitoneal injection of mice.

Toxin determination. Swiss strain white mice were challenged by intraperitoneal injection of 0.25 ml. of each filtrate. Death within 4 days was considered presumptive evidence of toxin in the sample. All presumptively toxic filtrates were confirmed by protection testing with polyvalent (ABC) *Cl. botulinum* antitoxin. Those filtrates which did not kill animals protected with an equal quantity of antitoxin but again destroyed unprotected control mice were considered to be confirmed positives.

RESULTS

Untreated Meat

Organoleptic Deterioration in Uninoculated Meats

Twenty uninoculated cans each of untreated beef and pork were sealed under nitrogen. Ten of each were frozen immediately after sealing, and the remaining cans were held up to 3 days at 99° F. Cans were removed and frozen as they swelled.

Contents of each can were used to prepare filtrates which were then inoculated into mice. Results, summarized in Table 1, show rapid deterioration of the test product held at 99° F. In addition, none of the filtrates were lethal, showing that the meats — both fresh and spoiled by indigenous putrefactive microorganisms — were not inherently toxic.

TABLE 1

Organoleptic and toxic characteristics of uninoculated pork and beef after three days incubation

	Holding temp.	No. of cans	No. of hard swells	Meat odor	No. of toxic cans
Pork	99° F.	10	10	Putrid	0
	−10°	10	0	Normal	0
Beef	99° F.	10	7 [1]	Off-Putrid	0
	−10°	10	0	Normal	0

[1] The contents of the 7 "hard swell" cans were putrid. The remaining 3 cans were only slightly swelled and the meat therein could best be described as "sour".

Cl. botulinum Inoculated Meat

99° F. Incubation. Results are shown in Table 2. All cans were normal after 4 hours; the meat had no off-odor and no toxin was detected. A definite off-odor was noted in cans incubated 8 hours. However, no toxin had been produced at this time. Within 24 hours all of the cans of pork had developed hard swells, the meat was highly putrid, and botulinum toxin was demonstrated. Most of the beef samples were putrid within 24 hours. All were hard putrid swells by the second day of incubation. Toxin was found only in definitely putrid cans.

70° F. Incubation. Table 3 lists the results. Spoilage, putrefaction and toxin production occurred much more slowly than at 99° F. Again, toxin formation was not demonstrated prior to obvious putrefactive breakdown.

TABLE 2

Organoleptic breakdown and toxin formation in inoculated meats at 99° F.

	Storage time	No. of cans tested	No. of hard swells	Meat condition	No. of toxic cans
Pork	0 hrs.	6	0	Normal	0
	4 hrs.	6	0	Normal	0
	8 hrs.	6	0	Off	0
	24 hrs.	6	6	Putrid	6
	2 days	3	3	Putrid	3
Beef	0 hrs.	6	0	Normal	0
	4 hrs.	6	0	Normal	0
	8 hrs.	6	0	Off	0
	24 hrs.	6	3	4 Putrid 2 Off	2 (Putrid only)
	2 days	3	3	Putrid	1
	5 days	3	3	Putrid	2
	9 days	3	3	Putrid	3

TABLE 3

Organoleptic breakdown and toxin formation in inoculated meats at 70° F.

	Storage time — Days	No. of cans tested	No. of hard swells	Meat condition	No. of toxic cans
Pork	0-2	30	0	Normal	0
	5	3	0	Off	0
	9	3	0	Off	0
	16	3	3	Putrid	1
	30	3	3	Putrid	3
Beef	0-2	30	0	Normal	0
	5	3	0	Normal	0
	9	3	0	Off	0
	16	3	0	Off	0
	30	3	3	Putrid	0

38° F. Incubation. No off-odors, swells or toxins were produced during 30 days of incubation at 38° F.

The data suggested three changes in protocol:

1. Toxin had been produced some time between the 8th and 24th hour at 99° F. Obviously, more intensive coverage of this period was indicated.

2. Since no organoleptic change or toxin production occurred in the product held at 38° F., this temperature was not included in subsequent experiments. Eighty-five degrees F., considered optimal for *Cl. botulinum* growth, was added as a temperature variable.

3. The coincidence of organoleptic breakdown in both inoculated and uninoculated product raised the question of whether toxin formation had occurred independently of product breakdown by the indigenous flora. This prompted the testing of product treated to destroy indigenous contaminants prior to addition of the *Cl. botulinum* inoculum.

Ethylene Oxide Treated Meat

Although the *Cl. botulinum* inoculum concentrations were equal to those used in the first experiment (10,000 per gram of meat), germination, growth and toxin production were strongly inhibited in the ethylene oxide treated meat. Although putrid swells and toxin occurred within 24 hours in the natural product, under the condition of ethylene oxide treatment, no swells or toxin could be detected until the 7th day of incubation. Of a total of 150 cans of product inoculated and incubated, only 3 became putrid during the course of the experiment: two of these contained detectable toxin. Toxin was not found in any of the non-putrid samples.

The results described above raised an interesting question on the effect of ethylene oxide on *Cl. botulinum* in fresh meat. Either the ethylene oxide absorbed by the meat had destroyed the spore inoculum or the meat had been rendered unfit as a germination and/or growth substrate for *Cl. botulinum*. This point was settled by subculturing the contents of 36 test cans into tubes of liver extract broth. Following 10-days' incubation at 85° F., these tubes were tested for toxin by the usual mouse inoculation procedure. Of a total of 36 cans tested in this manner, only 4 showed viable toxin-producing *Cl. botulinum* spores. These results demonstrated that residual ethylene oxide or its combination products had, in fact, destroyed most of the inoculum. Thus, the low level of spoilage and toxin production was attributable to the extremely low level of surviving spores.

Irradiated Meats

The low order of positive (toxic) samples in the ethylene oxide experiment demonstrated the necessity of utilizing some other technique to destroy the natural meat flora. Radiation sterilization was substituted for ethylene oxide perfusion in this experiment. The results are presented in Table 4. These data show, as with the untreated and ethylene oxide perfused meat, that toxin was found only in those samples which had undergone obvious putrefaction.

TABLE 4

Organoleptic deterioration and toxin production in irradiated fresh pork inoculated with Cl. botulinum

Incubation temperature	Storage time	No. of cans tested	No. of hard swells	Meat condition	No. of toxic cans
70° F.	0 hrs.	6	0	Normal [1]	0
	4 hrs.	6	0	Normal	0
	8 hrs.	6	0	Normal	0
	14 hrs.	6	0	Normal	0
	18 hrs.	6	0	Normal	0
	21 hrs.	6	0	Normal	0
	24 hrs.	6	0	Normal	0
	30 hrs.	6	0	Normal	0
	48 hrs.	6	0	Normal	0
	72 hrs.	6	0	Normal	0
	8 days	12	6	6 Putrid, 6 Normal	5 (Put. only)
85° F.	0 hrs.	6	0	Normal	0
	4 hrs.	6	0	Normal	0
	8 hrs.	6	0	Normal	0
	14 hrs.	6	0	Normal	0
	18 hrs.	6	0	Normal	0
	21 hrs.	6	0	Normal	0
	24 hrs.	6	0	3 Norm. 2 Off, 1 Put.	0
	30 hrs.	6	0	5 Norm. 1 Off	0
	48 hrs.	6	3	3 Norm. 3 Put.	3 (Put. only)
	72 hrs.	6	6	6 Putrid	6
	8 days	6	6	6 Putrid	6
99° F.	0 hrs.	6	0	Normal	0
	4 hrs.	6	0	Normal	0
	8 hrs.	6	0	Normal	0
	14 hrs.	6	1	4 Norm. 1 Off, 1 Put.	0
	18 hrs.	6	1	3 Norm. 1 Off, 2 Put.	0
	21 hrs.	6	3	3 Norm. 1 Off, 2 Put.	0
	24 hrs.	6	3	1 Off, 5 Put.	5 (Put. only)
	30 hrs.	6	6	6 Putrid	6
	48 hrs.	6	6	6 Putrid	6

[1] The irradiated meat had a distinctive "aged" odor. "Off" refers to meat which showed a sour, "fermented" odor and might conceivably be acceptable to a consumer. "Putrid" described those samples which were organoleptically repulsive.

A summation of the results of all three experiments is presented in Table 5.

TABLE 5

Summary of data on toxin production and organoleptic unacceptability in anaerobically packed fresh meat inoculated with 10,000 Cl. botulinum spores per gram

	Incubation temperature °F.			
	70	85	99	Totals
Putrid Cans	12	17	65	94
Putrid Cans Toxic	8	16	51	75
Putrid Cans Not Toxic	4	1	14	19
% Toxic and Putrid	100	100	100	100
% Non-Toxic and Putrid [1]	33	5	21	20
% Toxic Not Putrid	0	0	0	0
"Off" Cans	18	3	17	38
"Off" Cans Toxic	0	0	0	0

[1] The difference between the percentages of non-toxic and putrid containers at 70°-85° and 99° cannot be considered as significant in view of the small numbers of putrid cans at 70° and 85° F.

DISCUSSION

When one correlates toxin development with obvious putrefactive degradation, it can be seen that no single instance of toxicity was observed in a product sample which could be considered edible. Table 5 shows that while all toxic samples were also putrid, all putrid samples were not necessarily toxic. A total of 75 toxic samples were detected. Ninety-four samples, including the 75 toxic ones, were obviously putrid. In addition, the contents of 38 cans underwent enough bacteriological activity as to be readily identified as "off". These 38 samples are significant only as examples of a "twilight" zone of spoilage which might or might not be considered a sign of danger by the consumer. None contained detectable toxin. The experimental results are, of course, not presented as "absolute proof" that botulinum toxin cannot occur in organoleptically sound fresh meat. Tanner *(8)* and Dack *(1)*, pointing out that *Cl. botulinum* ordinarily causes very offensive decomposition in food products, caution that contamination with a non-proteolytic strain would constitute a hazard. It should be recalled, however, that fresh meats are essentially different from thermally processed products in that they normally are contaminated with a wide variety of genera capable of anaerobic growth *(6)*. The possibility of non-proteolytic *Cl. botulinum* organisms occurring alone in a package of fresh meat is infinitely small. The data show that the nature of the product and the growth characteristics of *Cl. botulinum* combine to build into the system a remarkably efficient safety device. This was found to be true in meat containing natural contamination as well as in meat largely denuded of its indigenous flora by ethylene oxide gas and by ionizing radiation.

SUMMARY

Data have been presented upon the rate of toxin formation and putrefactive breakdown in fresh meats seeded with spores of *Cl. botulinum* and packed under anaerobic conditions. Comminuted pork and beef were utilized both in the natural state and denuded of indigenous contaminating flora

by means of ethylene oxide gas perfusion or by the use of ionizing radiations.

In no case did detectable toxicity develop prior to obvious organoleptic putrefactive breakdown. In some instances putrefaction occurred without the development of toxicity. In other instances an "off" or "sour" type of organoleptic breakdown occurred but in none of these was toxicity detected. It appears that the nature of these products combined with the characteristics of *Cl. botulinum* form a system which can be remarkably efficient in warning the consumer of possible hazard in the event they are grossly mishandled.

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VARIATIONS IN TENDERNESS WITHIN THREE MUSCLES FROM BEEF ROUND[a, b, c]

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Designing experiments to study treatment effects upon tenderness in meat requires the consideration of several factors. In addition to differences in tenderness among animals and among muscles in individual carcasses, there is a range in tenderness within the muscles themselves *(3, 4, 6, 7, 8, 11)*. The effect of muscle location upon tenderness becomes important when steaks are allocated to different treatments.

Several studies have been carried out on location differences in the ribeye muscle *(4, 8, 11)* but very few have dealt with variations in the less tender muscles from the round *(6, 8)*. Because these muscles have larger amounts of connective tissue *(5)* they have a greater need for tenderization. Data on variations in shear strength are available for the *biceps femoris (8)*, *semimembranosus*, and *adductor (6)* muscles. The *biceps femoris* increased in shear strength from 7.5 to 10.8 pounds from the anterior to the posterior. The *semimembranosus* tested at 10 positions varied significantly in shear value and was less tender at the center than the extremities. The *adductor* muscle was tested at 5 positions and did not vary significantly.

Shear values have been used extensively to indicate the relative tenderness of meat. They do not reflect as small differences, however, as taste panel scores, at least not in the *longissimus dorsi* muscle. It is possible, in fact, that they reflect only a portion of the changes in tenderness which may occur *(2, 10, 11)*. Recent studies on tenderizing procedures and their effect upon the initial tenderness and residue of beef steaks have emphasized the need to differentiate among the factors contributing to tenderness.

A previous report from this laboratory *(3)* indicated that panel scores for initial tenderness in the *semimembranosus* muscle were greatest at the anterior and decreased gradually throughout the muscle *(3)*. Since that time two other muscles from the round have been studied with additional data obtained on shear strength and panel evaluations for initial tenderness and residue.

MATERIALS AND METHODS

Semimembranosus, *semitendinosus* and *biceps femoris* muscles were removed from U. S. Good beef rounds 48 to 72 hours post-slaughter. The *semitendinosus* and *biceps femoris* muscles were obtained from the same 4 animals; the *semimebranosus* muscles were from a different group of 6 animals. They were sliced into ¾ in. steaks on a standard model U. S. slicing machine and wrapped in LSAT-300 cellophane. The steaks were then frozen and stored at −10° F. while taste panel evaluations were in progress.

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[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, Illinois, May 26, 1958.

[b] Journal paper No. 161 from the American Meat Institute Foundation.

[c] The authors wish to acknowlege the assistance of Paul D. Brown, Division of Food Technology, American Meat Institute Foundation, in obtaining the shear values in this study.

Six *semitendinosus* muscles were sliced into ½ in. thick steaks for freeze-drying [4]. Dried steaks were stored in cans under 15 inches vacuum. They were rehydrated in 2.0% sodium chloride solution for 30 minutes immediately before cooking. Frozen steaks were thawed for 18 to 22 hours at 40° F. Both fresh and rehydrated steaks were broiled by a standard procedure described elsewhere *(12)*.

Steaks were numbered from the anterior (pelvic) end of the muscles; end steaks not large enough to serve a taste panel of 8 were discarded. The *semimembranosus* provided 8 usable steaks, the *biceps femoris* and *semitendinosus* each provided eleven. The dehydrofrozen *semitendinosus* muscles provided 15-22 steaks; data on these muscles were adjusted to represent 15 similarly located positions.

Samples for panel evaluation were approximately ½ x ¾ x ¾ in. in size from the fresh steaks and ½ x ½ x ½ in. from the dehydrofrozen. The panel was comprised of 8 persons experienced in judging tenderness of meat. The same judges served throughout the study and each always received his sample from the same position within each steak. Tenderness ratings consisted of 2 parts *(10)*; tenderness which was defined as the initial impression of softness and residue which referred to the amount of residual material remaining after chewing. Tenderness and residue rating scales ranged from 1 to 10; tenderness scores increased with increasing tenderness and residue scores increased as the residue diminished except for the dehydrofrozen steaks where residue scores were reversed. Shear strength measurements, using the Warner-Bratzler shear apparatus, were made on 5 cores, ½ in. in diameter, cut parallel to the muscle fibers of the *semimembranosus* and *biceps femoris* muscles. Data were analyzed for over-all variance and individual variance due to muscle location *(1, 9)*.

RESULTS AND DISCUSSION

Wide variation in both tenderness and residue scores was noted throughout the individual muscles (Figures 1, 2). Shear strength values for the *semimembranosus* and *biceps femoris* muscles (Figures 3, 4) followed the same trends. Tenderness and residue scores varied inversely, low residue scores being associated with low shear strength and greater initial tenderness.

All muscles showed significant location, animal and judge differences (Table 1). Judge variation included tenderness differences within individual steaks as well as scoring differences among the eight judges on the panel. Variation due to the interaction between judges and locations was significant only in the *semimembranosus* muscle where the judges did not agree upon how the tenderness varied among steak locations. An evaluation of the judges' performance in tenderness scoring during the early part of this study was reported elsewhere *(3)*. Since the judges were able to reproduce very closely their tenderness scores on longitudinally adjacent sections of meat from individual steaks, it is possible that a greater difference within each steak in relation to its location in the *semimembranosus* muscle was responsible for the significance of the interaction. The *semimembranosus* muscle also showed the greatest steak-to-steak variations (Figures 5, 6). These figures were obtained by arranging the steaks in order of decreasing tenderness, increasing residue, and shear strength. The more uniform steaks (differences having a lesser significance than $P = 0.05$) were bracketed. Adjoining steaks in the *semimembranosus* muscle varied significantly ($P = 0.001$) in both tenderness and residue between steaks 6 to 9 and 2 to 5. The most abrupt changes occurred between steaks 4, 5, and 6, and were least abrupt in the posterior portion of the muscle which was less tender and had more residue. Shear values showed lesser variation, but steaks 2 to 4 and 7 to 9 were quite different ($P = 0.001$).

[4] These steaks were provided by the Animal Products Division, Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois.

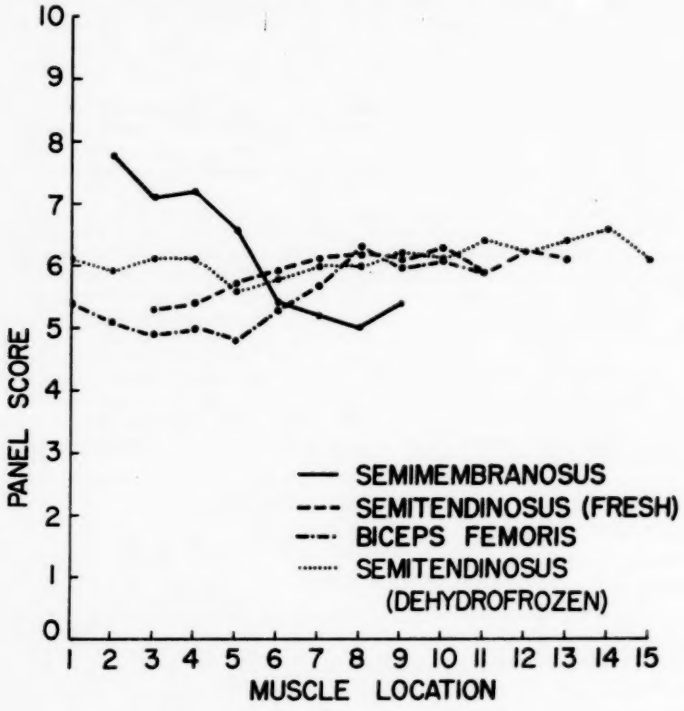

Figure 1. Muscle variation in tenderness.

Tenderness and residue differences along the other muscles were smaller. The fresh and dehydrofrozen *semitendinosus* muscles were the most uniform. Steaks from the posterior of the fresh muscle (locations 6 through 13) differed significantly in neither tenderness nor residue. The residue scores for dehydrofrozen *semitendinosus* steaks showed greater variance than did the tenderness scores and both displayed a different pattern of variance than in the fresh muscles. The anterior portion (locations 2 to 6) of the *biceps femoris* was more uniform than the posterior, but was less tender and had more residue. Residue and tenderness differences occurred to a similar extent. Adjoining steaks from either the *biceps femoris* or *semitendinosus* muscles may be compared directly for tenderness or residue. Steaks from the anterior half of the *biceps femoris* or the posterior half of the *semitendinosus* may be distributed at random when necessary.

Shear strength values differed significantly among steaks taken from different locations in the *semimembranosus* muscle, but not in the *biceps femoris* (Table 2). Further analysis of the data to compare steak locations (Figure 5) indicated that the *biceps femoris* was quite uniform in shear strength while the *semimembranosus* muscle differed markedly. The panel and shear values for the *biceps femoris* (Figure 4, 5) show similar trends throughout

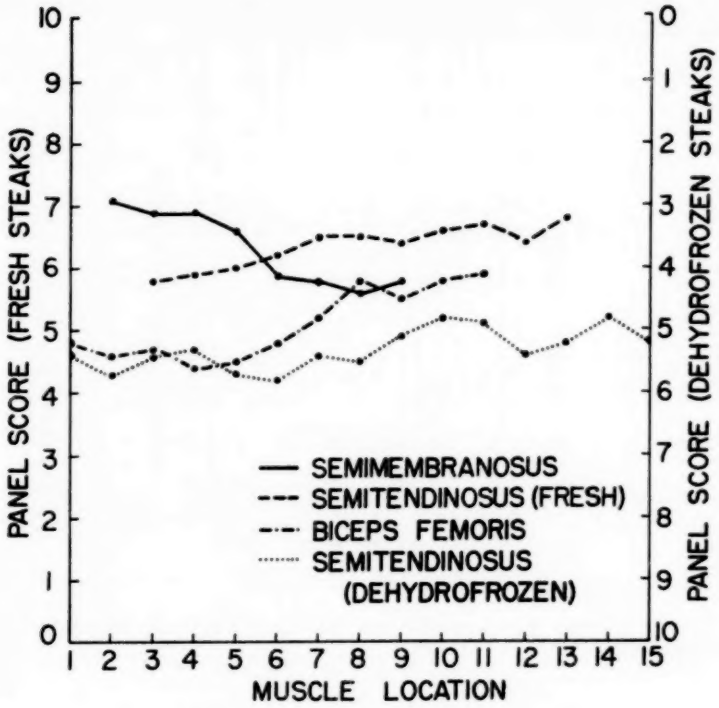

Figure 2. Muscle variation in residue.

the length of the muscle, but the panel score variations were more significant. This was true also for the *semimembranosus* muscle (Figure 3, 5). Panel measurements of tenderness were significant for smaller differences than shear strength values.

SUMMARY

Panel evaluations for tenderness and residue are reported for the *biceps femoris, semimembranosus, semitendinosus* and dehydrofrozen *semitendinosus* muscles. Shear strength measurements were made on *biceps femoris* and *semimembranosus* muscles.

All the muscles varied significantly in tenderness and residue throughout their length. The changes in the *biceps femoris* and both types of *semitendinosus* muscle were less abrupt than in the *semimembranosus*. The anterior half of the *biceps femoris* and the posterior half of the *semitendinosus* were both quite uniform. Shear strength varied significantly in the *semimembranosus*, but not in the *biceps femoris* muscles. While the shear values reflected changes in tenderness and residue scores, they were less sensitive to differences within the muscles. The *biceps femoris* and *semitendinosus* muscles being

more uniform in tenderness and residue than the *semimembranosus*, were more suitable for the study of tenderness treatments.

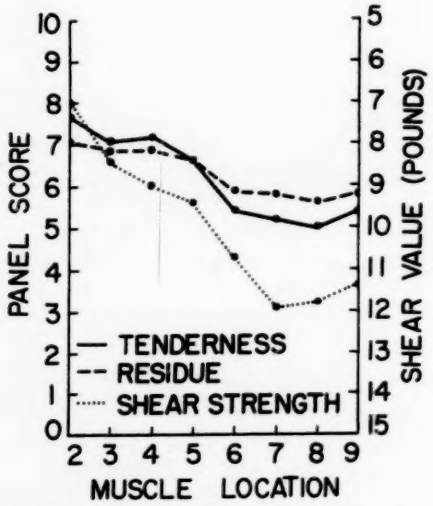

Figure 3. Shear values, residue, and tenderness scores for semimembranosus muscles (mean score for 6 animals).

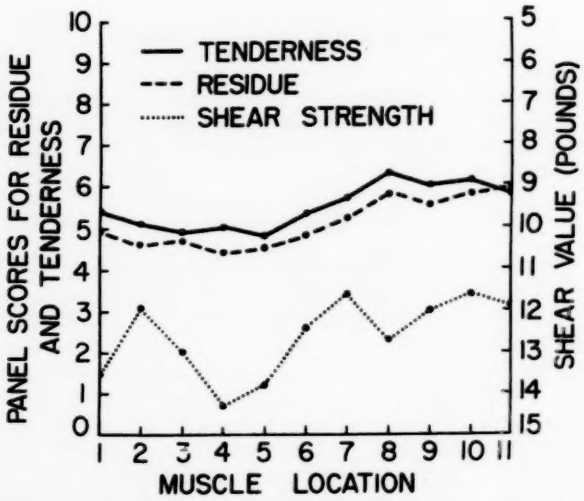

Figure 4. Shear values, residue, and tenderness scores for biceps femoris muscles (mean score for 4 animals).

TABLE 1

Analyses of variance in tenderness and residue scores

Muscle	Sources of variation	df	Tenderness		Residue	
			Mean squares	"F" value	Mean squares	"F" value
Semimembranosus	Animals (A)	5	50.44	54.83 ***	23.93	29.54 ***
	Judges (J)	7	29.56	32.13 ***	18.00	22.22 ***
	Locations (L)	7	56.11	60.99 ***	36.78	45.41 ***
	A x J	35	1.71	1.86 *	2.10	2.59 ***
	A x L	35	2.36	2.56 ***	2.52	3.10 ***
	J x L	49	2.27	2.47 ***	2.73	3.37 ***
	A x J x L	245	0.92		0.81	
Semitendinosus (fresh)	A	3	75.44	72.54 ***	55.66	92.77 ***
	J	7	67.61	65.01 ***	76.12	126.87 ***
	L	10	3.64	3.50 ***	3.41	5.68 ***
	A x J	21	3.96	3.81 ***	4.89	8.15 ***
	A x L	30	2.40	2.31 ***	4.89	8.15 ***
	J x L	70	1.22	1.17	0.82	1.37
	A x J x L	210	1.04		0.60	
Biceps femoris	A	3	99.91	48.03 ***	58.18	32.68 ***
	J	7	29.79	14.32 ***	74.59	41.90 ***
	L	10	9.12	4.38 ***	10.53	5.92 ***
	A x J	21	5.82	2.80 **	5.42	3.04 ***
	A x L	30	5.90	2.84 ***	2.42	1.36
	J x L	70	2.02	0.97	1.35	0.76
	A x J x L	210	2.08		1.78	
Semitendinosus (dehydrofrozen)	A	5	30.31	28.59 ***	40.41	31.82 ***
	J	7	53.10	50.09 ***	52.44	41.29 ***
	L	14	3.21	3.03 ***	5.34	4.20 ***
	A x J	35	3.79	3.58 ***	5.37	4.23 ***
	A x L	70	3.60	3.40 ***	2.84	2.24 **
	J x L	98	0.92	0.87	1.07	0.84
	A x J x L	490	1.06		1.27	

* Significant at the 5% level.
** Significant at the 1% level.
*** Significant at the 0.1% level.

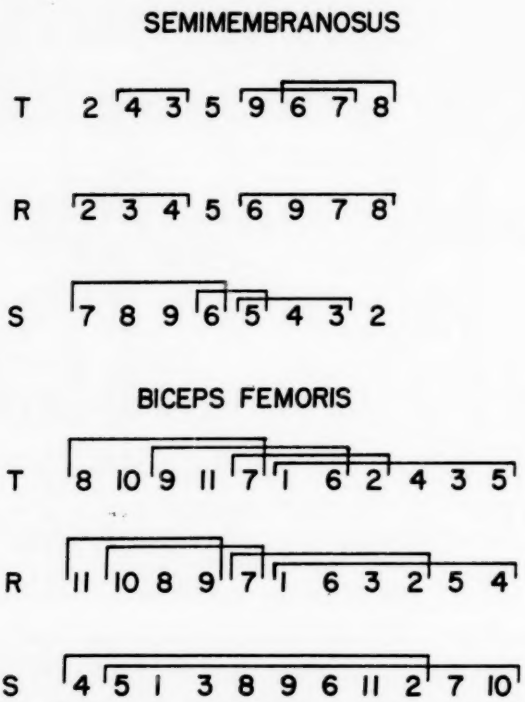

Figure 5. Variation in tenderness, T, residue, R and shear strength, S (P = 0.05) in semimembranosus and biceps femoris muscles.

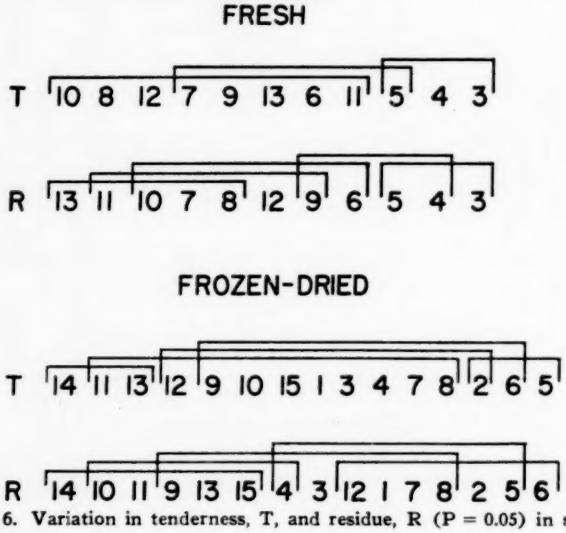

Figure 6. Variation in tenderness, T, and residue, R (P = 0.05) in semitendinosus muscles.

TABLE 2
Analyses of variance in shear values

Muscle	Sources of variation	*df*	Mean squares	"F" value
Semimembranosus	Animals (A)	5	100.45	15.17 ***
	Locations (L)	7	94.61	14.29 ***
	A x L	35	4.34	0.66
	Residual	192	6.62	
Biceps femoris	A	3	177.96	11.32 ***
	L	10	16.35	1.04
	A x L	30	15.53	0.99
	Residual	176	15.72	

*** Significant at the 0.1% level.

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A METHOD FOR MEASURING THE RHEOLOGICAL PROPERTIES OF EGGS*

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(Manuscript received May 31, 1958)

Separation of eggs into grades on the basis of interior quality in an accurate, objective and reproducible manner is desirable in market channels. To accomplish this separation, the internal condition of the intact hen's egg should be evaluated without shell damage. Methods and principles that could be used for the automatic grading of eggs have been investigated as a cooperative project with the Poultry Research Branch, Agricultural Research Service. These techniques include radio frequency *(9)*, ultrasonics and microwaves *(7)*, light transmission *(3)*, fluorescence *(8)*, and rheology. This discussion deals with rheology.

Rheology is the study of flow and deformation of matter *(1, 5)*. Rheological properties may be characterized as plastic, pseudo-plastic, fluid, consistent, viscous, dilatant, thixotropic, rheopectic or combination effects. The rheological properties of Newtonian liquids may be characterized rather easily by viscosity measurements. Numerous methods and devices such as the Ostwald Viscometer *(1, 5, 6)*, falling sphere, aperture and torsion have been used to measure viscosity. Egg albumen components, each of a different viscosity, are difficult to characterize. Each of the various components of an egg could be characterized for their rheological properties or the egg may be considered as a unit and its total properties measured. In the study reported here, only the total characteristics of an egg are considered, since the purpose of the study was to develop a means of measuring the albumen quality of an intact egg.

Wilcke *(12)* measured the combined viscosity of all the components of an egg with a torsion pendulum by measuring the damping in pendulum oscillations caused by the egg. He determined the correlation of this damping factor with yolk movement, yolk shadow intensity, percent thin white, viscosity of thin white, volume of thin white, and volume of thick white. The highest correlation coefficient (only 0.24) was with yolk movement. The torsion pendulum type of measurement was analyzed by Atanasoff and Wilcke *(2)* in 1937, but since that time no further effort has been devoted to this technique. These workers used only a very limited range of oscillation frequency on their torsion pendulum and did not attempt a correlation with albumen quality as now defined by Brant, Otte, and Norris *(4)*. The torsion pendulum type of measurement still appears to offer the best means of indicating the total visco-elastic properties of an egg. Therefore, a new evaluation of this technique was made.

MATERIALS AND METHODS

A torsion pendulum apparatus was constructed using Wilske's apparatus as a guide. Provisions were made to vary the oscillation frequency from 0.1 to 100 cycles per second

* Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 27, 1958.

(c.p.s.) and to measure the damping factor automatically. Figure 1 is a drawing of the torsion pendulum. The pendulum consisted of a light weight metal cage which serves as the egg holder and is suspended between equal lengths of steel piano wire. Oscillation frequencies were varied by changing the suspension wire length, the moment of inertia or both. The adjustable weight distance from the axis of rotation was varied up to 4 inches. It was necessary to use a suspension wire size that would show the greatest effect by the egg damping. For a particular frequency, a wire was selected within the diameter range 0.010 to 0.050 inch. An oscillation frequency obtained by varying the wire length, the moment of inertia, or a combination of these factors yielded the same results. For calibration at a given frequency and as a periodic check, a wax-filled glass egg was placed in the holder and the frequency determined.

The damping factor was measured by determining the number of oscillations occurring during a rotational angle change from approximately 20 degrees to approximately 10 degrees. A focused light beam was reflected from the mirror, Figure 2, on the egg holder. Rotational motion of the mirror traversed the light beam to a photocell. As the light beam traversed the sensing area of the photocell, one count was recorded electronically and the total count recorded as the damping factor. This photocell was located in such position as to insure the most accurate counting of the oscillations. To start the system oscillating, the cage was moved to the starting position and engaged by the release mechanism. Upon release, rotational oscillations of the holder about the suspension axis traversed the light

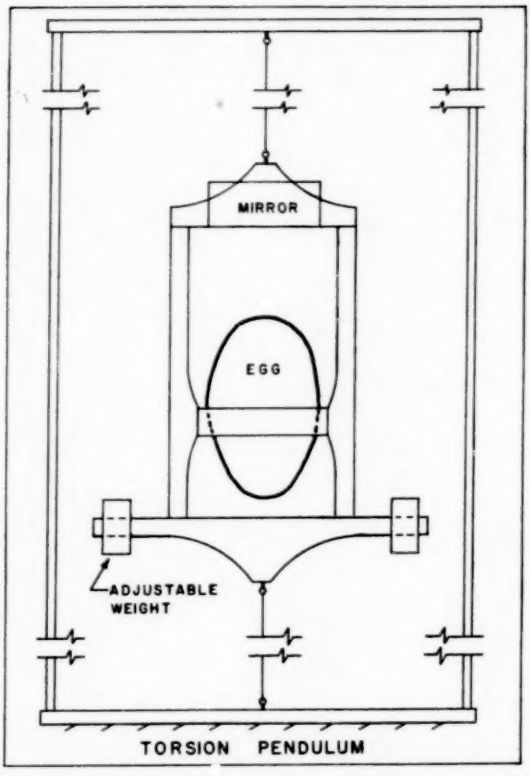

Figure 1. Torsion pendulum arrangement for measuring egg characteristics. The egg is held in a rubber-gasketed aluminum framework supported between steel wires.

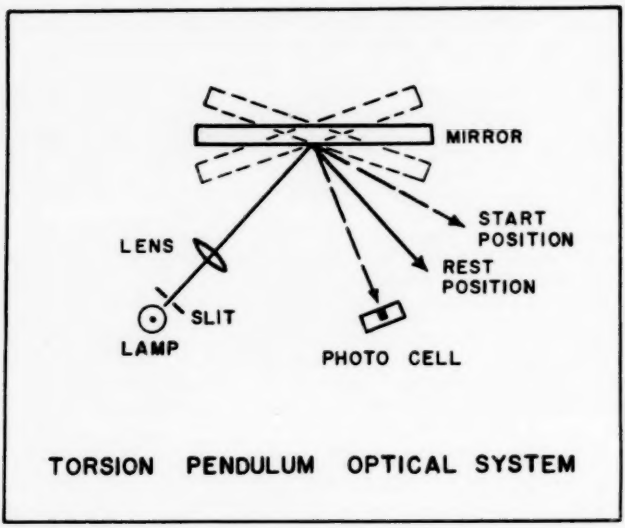

Figure 2. Optical system with collimating slit and focusing lens. Light beam traverses the angle between the start position and photocell.

beam through the angle between the starting position and the photocell. The light beam activated the photocell during each oscillation until the "damping effect" reduced the angle of rotation to a point where the photocell was not affected.

The number of oscillations for a given angular displacement is a function of the system damping properties. Damping occurs when the direction of rotation of a container holding a liquid is suddenly reversed. The finite layers of liquid nearest to the container wall change direction, but the liquid away from the wall continues momentarily in the original direction. This resistance to change of direction is the damping action which decreases the angle of rotation. At a given frequency, it was observed that damping is greater for thick or viscous liquids (glycerol) than for less viscous liquids (water). This type of damping is absent when solids are rotated in a like manner. Therefore, the number of oscillations is greater if a solid is placed in the egg holder and oscillated than when a liquid is in the egg holder.

RESULTS AND DISCUSSION

Viscosities of water-glycerol solutions were determined with an Ostwald Viscometer. These solutions were then placed in a glass egg and the damping factor measured. These values were plotted, damping factor vs. viscosity on a semi-log scale, Figure 3. Since this technique is reproducible, viscosity of a similar solution may be determined easily by relating its damping factor to this calibration curve.

The behavior of eggs oscillated at different frequencies was also studied. At various frequencies, 0.2 to 30.0 c.p.s., plots were made for damping factor vs. Haugh units (egg grade based on the "broken-out" albumen quality). The slope of the regression line changed from negative to positive values at approximately one cycle per second. A frequency between two and three c.p.s. resulted in the best correlation between damping factor and quality.

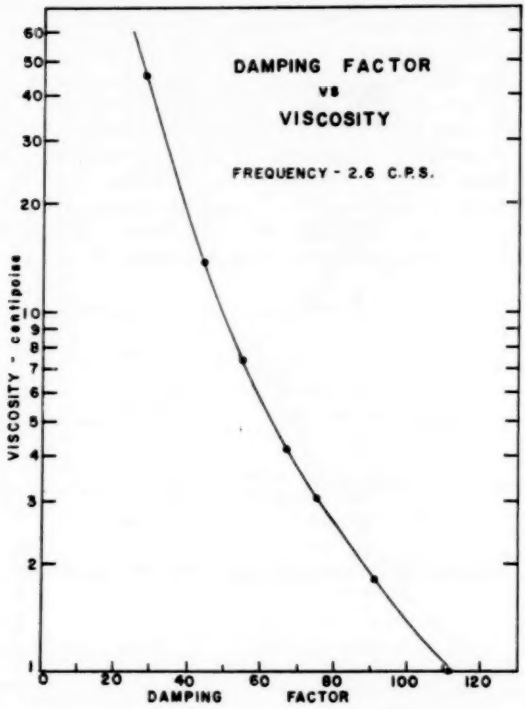

Figure 3. Viscosity calibration curve obtained with glycerol-water solutions in a glass egg.

Negligible effect on the damping factor was contributed by shape and positioning of the egg in the holder. Egg weight, however, had considerable effect. Therefore, a correction curve relating damping factor to egg weight was plotted, Figure 4. This plot indicates that, for each egg, a proportionate value should be subtracted from the damping factor if the weight is less than 20 ounces per dozen or a proportionate value added if the weight is greater than 20 ounces per dozen.

A Haugh unit vs. damping factor correlation for 318 eggs produced a coefficient $r = 0.74$ and a regression equation $y = 9.35 + 0.144\ X$, Figure 5. The damping factor for this group of eggs was measured at 2.6 c.p.s. using two lengths of suspension wire 11.5 inches long with a diameter of 0.029 inch. It may be noted from Figure 5 that the damping factor for eggs of the same Haugh unit values (corrected for weight difference) are inconsistent. Factors that appeared to contribute to this inconsistency were temperature, air cell size, egg shape, yolk size, yolk location, yolk sticking and amount of albumen remaining in the shell. Evaluation of these factors failed to explain this variation.

It has been reported that eggs produced by the same hen have consistently

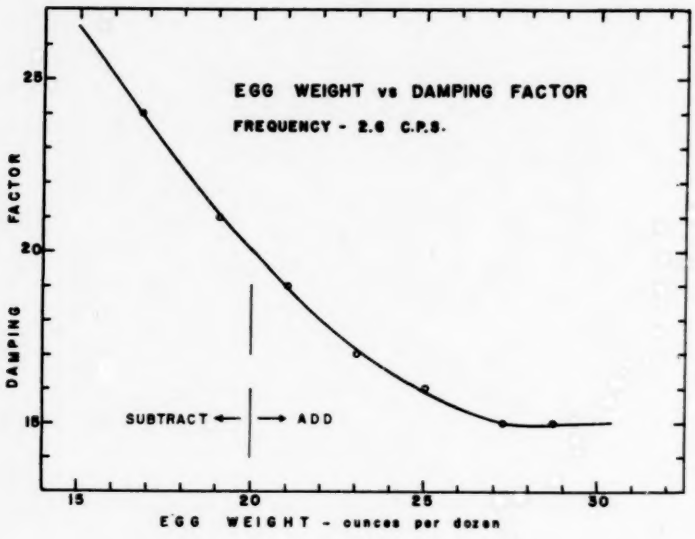

Figure 4. Variation of damping factor with egg weight.

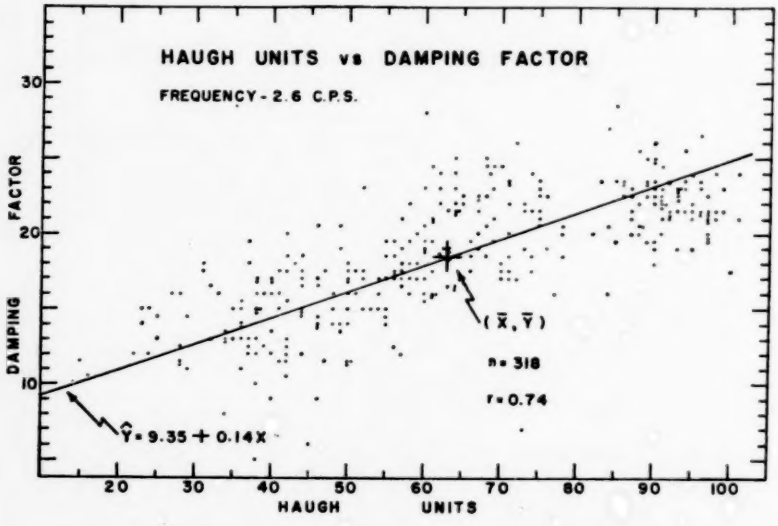

Figure 5. Scatter diagram for eggs varying in albumen quality.

similar interior quality, shell quality, shape, and interior characteristics. Therefore, it was decided to investigate whether damping factor of eggs from the same hen was also consistent. The breakout scores of eggs from a group of hens were recorded. On this known history basis, a number of hens were selected so that eggs of different albumen qualities were represented. The damping factor for each egg from these hens was recorded at the age of one day. The eggs were refrigerated for 7 days. At this time, the damping factor was measured again and the breakout quality recorded in Haugh units. Even though the quality of eggs from the same hen was consistent, the damping factor for 1-day-old eggs varied as much as 22% between eggs from the same hen laid on consecutive days, Figure 6. This

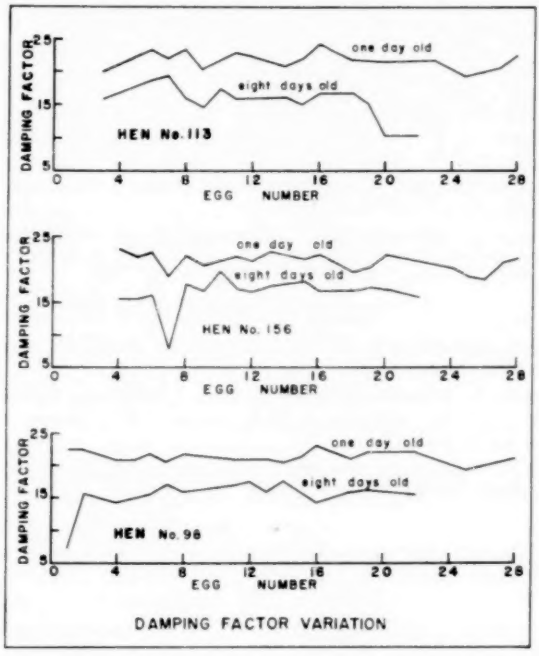

Figure 6. Variation of damping factor characteristics for eggs from the same hen.

variation in damping factor was much greater on the 8-day-old-eggs, but this may be the result of stuck yolks in some of the eggs.

CONCLUSIONS

The torsion pendulum provides a means of making simple and reproducible measurements of viscosity. It can be calibrated for a desired frequency, the calibration maintained or it can be varied easily. These studies indicate that although this technique can be used to separate low quality eggs

from those of high quality, it is not sufficiently accurate for purposes of grading eggs. Results of the glycerol viscosity determinations indicate that there may be a use for this type of device as a laboratory tool.

Acknowledgment

The authors appreciate the cooperation of Dr. A. W. Brant and staff of the Poultry Research Branch, ARS, in supplying eggs of varying quality and assisting in analysis and interpretation of data.

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METHOD FOR DETERMINING THE SUB-SIEVE PARTICLE SIZE DISTRIBUTION OF PULVERIZED SUGAR[a]

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(Manuscript received May 31, 1958)

The sub-sieve particle size distribution of pulverized sugars has long interested many manufacturers and users of pulverized sugars. Lack of a practical method for determining this distribution has hindered, unfortunately, possible product developments that might otherwise have occurred. Likewise, knowledge of the size distribution in the sub-sieve range may influence economies in equipment design and in control of processing operations. Such knowledge may be of value in determining the commercial utilization of the product. It is well known, for example, that pulverized sugar particles measuring 45 μ or larger impart a gritty texture in cream candies and icings.

Today, as well as historically, the primary method used for size analysis for sugars is the standard screen analysis *(3)*. In a screen analysis the sugar sample is put through a number of sieves of successively smaller mesh size. The weight fraction which passes one sieve and is retained on the next smaller size is determined. The impracticality of obtaining satisfactory results with sieves of very small openings has limited screen analyses to particles of 44 μ or larger. Interestingly enough, the largest fraction of all commercially available pulverized sugars lies in the sub-sieve range.

The present investigation was undertaken to develop a relatively simple and accurate method whereby particle size distributions of pulverized sugars could be determined in the sub-sieve range. Although several methods are available for sub-sieve particle size measurements, they are either very tedious, specialized, or require elaborate equipment *(5, 6, 8)*. The Andreasen pipette sedimentation method was selected for this work as it is capable of yielding accurate results and requires rather inexpensive, simple apparatus *(1, 2, 4, 7, 9)*. Sedimentation methods, in general, have proven very satisfactory on many water insoluble materials such as plastics, coal, clay, starches, cements, powdered metals, insecticides, and ground minerals, where water is used as the suspending medium. However, the sedimentation principle has seldom been used on anything as water soluble as sugar, where the presence of any water would be a definite hindrance to the analysis.

In the sedimentation method, it is necessary to begin with a homogeneous mixture of sugar particles and a liquid medium. The concentration of particles in the medium will decrease with time at any fixed depth below the surface. In the proposed method the particle concentration in a 10 ml. aliquot, approximately 20 cm. below the surface of the suspension, is periodically determined. This concentration becomes a calculable function of particle size in Stokes' Law and the Andreasen pipette apparatus shown in Figure 1 is used to determine this concentration.[b] The cylinder portion (capacity, ap-

[a] Presented at the Eighteenth Annual Meeting of the Institute of Food Technologists, Chicago, May 26, 1958.

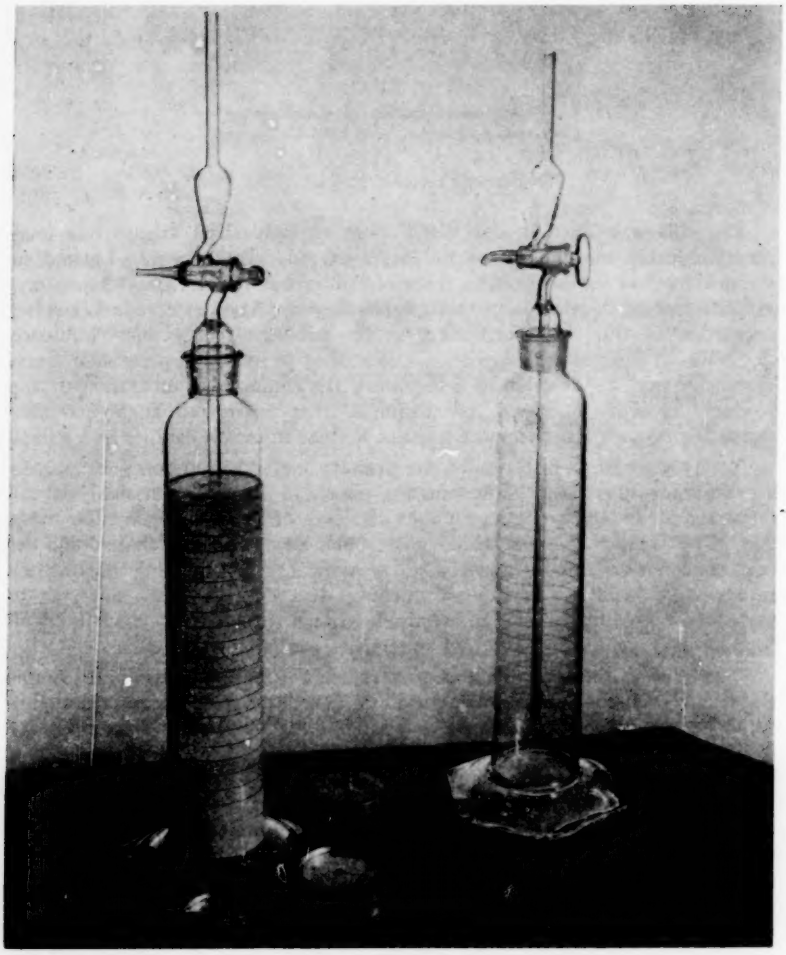

Figure 1. Andreasen pipette assembly for particle size distribution measurements.

proximately 550 ml.) is calibrated in centimeters from the point at which all samples are withdrawn to the starting solution level near the top of the cylinder. The pipette has a bulb capacity of 10 ml. A capillary stem extends down to the zero calibration mark on the cylinder.

MATERIALS AND METHODS

Isopropyl alcohol. Since even the slight moisture content of commercial isopropyl alcohol appeared to influence the degree of agglomeration of sugar particles in the dis-

[b] Obtainable from Scientific Glass Apparatus Co., Inc., Bloomfield, New Jersey.

persion medium, all isopropyl alcohol used for this purpose was redistilled over calcium oxide.

Dispersion medium. About 5 g. of granulated sugar and 5 ml. of dispersing agent (Twitchell 8240)[e] are added to approximately 2½ liters of freshly distilled isopropyl alcohol. This dispersion medium is placed in a stoppered bottle and mixed intermittently during a one-hour interval to insure saturation with sucrose. It can be stored indefinitely in this condition and is filtered through Whatman No. 1 filter paper just prior to use.

PROCEDURE

a. The tare weight of several aluminum weighing dishes is determined. The number of dishes will depend on the number of size fractions to be determined.

b. Approximately 7 g. of the pulverized sugar for which the particle size distribution is required is weighed out. This sugar and roughly 300 ml. of dispersion medium are placed in a milkshake type mixer cup and mixed for 15-20 minutes.

c. This suspension is then transferred to an Andreasen cylinder. The mixer cup is rinsed with small portions of dispersion medium and added to the cylinder. The level of the suspension in the cylinder is brought to the 20 cm. mark with additional dispersion medium.

d. The dry Andreasen pipette is inserted into the cylinder containing the suspension and the contents are thoroughly mixed for at least one minute by repeated inversion of the cylinder. Immediately upon stopping, the suspension is drawn up into the pipette and the level is adjusted to exactly the 10 ml. mark on the pipette. No more than 15 seconds should elapse from the time mixing is stopped until the level is adjusted to the 10 ml. graduation mark on the pipette. All calculations are based on this zero time sample and, therefore, extra effort is made in obtaining the sample very accurately.

The stopcock is then turned to the position where the 10 ml. aliquot is emptied into a tared aluminum dish. The Andreasen pipette is then rinsed with 5 ml. of regular (not saturated) isopropyl alcohol with this rinse material being collected in the same aluminum dish.

e. The Andreasen pipette is then rinsed with a second portion of regular isopropyl alcohol and drain dried for a few minutes.

f. The stopcock is again closed, the pipette is placed in the cylinder, and the contents are again mixed by repeated inversion of the cylinder for one minute. As soon as the mixing process is stopped, the cylinder is set down and a stopwatch is started.

g. The level of the suspension is read off of the graduated cylinder and recorded.

h. After settling for a definite time interval, which will depend on the size distribution that is desired, another 10 ml. sample is removed as described previously. This sample is discharged into another tared aluminum dish followed by a 5 ml. regular isopropyl alcohol wash of the Andreasen pipette. Without removing the Andreasen pipette from the cylinder, the stopcock is turned to the position where the suspension remaining in the capillary portion of the pipette drains back into the cylinder.

i. Step g and h are repeated at different time intervals.

j. For a blank determination, 10 ml. of dispersion medium is transferred to a tared aluminum dish, using the Andreasen pipette, followed by a 5 ml. regular isopropyl alcohol wash.

k. The alcohol is evaporated from all the aluminum dishes. After allowing the dishes to equilibrate to room temperature, they are weighed and the particle size distribution is calculated.

CALCULATIONS

Like other particle size methods based on sedimentation, the present one depends on Stokes' Law which, in effect, states that small spheres falling freely through a suitable medium maintain a velocity which is proportional to the square of their diameter. Although pulverized sugar particles are not small spheres, satisfactory approximations are obtained using Stokes' Law.

$$d^2 = \frac{3h\ n\ 10^7}{(p_1 - p_2)\ gt}$$

[e] Avalable from Emery Industries, Inc., Cincinnati 2, Ohio.

h = depth at which sample is taken (cm.)
n = viscosity of dispersion medium at 25° C. (0.02066 poises) (determined with Ostwald Viscosimeter)
p_1 = density of solid material, i.e., sucrose (1.5877 g./cc.)
p_2 = density of dispersion medium at 25° C (0.7860 g./cc.) (determined with pycnometer)
g = acceleration due to gravity (980 cm./sec.^{2})
d = largest particle diameter in any aliquot of the settling suspension (microns)
t = time of settling (minutes)

Since n, p_1, p_2 and g are constants for this particular determination, the equation can be simplified to:

$$d^2 = \frac{(3)\ (0.02066)\ (10^7)\ (h)}{(1.5877\text{-}0.7860)\ (980)\ (t)} = \frac{788.9h}{t}$$

Determination of largest particles in an isolated fraction

The level in the Andreasen cylinder at which all samples are taken is fixed, but the height (h) of the suspending medium above this level varies as each succeeding sample is removed. After any particular time of settling (t), the largest particle diameter (d) that has not passed the level at which the sample is removed can thus be calculated.

Example No. 1: After 10 minutes settling, all particles larger than d μ will have settled a distance of 20.3 cm, or more (see Table 2)

$$d = \sqrt{\frac{(788.9)\ (20.3)}{10}} = 40 \text{ microns}$$

Percentage by weight of particles finer than the calculated fraction size =

$$\frac{\text{Weight of sugar in suspension at time t}}{\text{Weight of sugar in suspension at time o}} \times 100$$

Example No. 1: Percentage by weight of particles finer than 40 microns =

$$\frac{93.6}{111.1} \times 100 = 84.2\%$$

(see Table 2)

RESULTS AND DISCUSSION

The accuracy and reliability of the sedimentation method depend upon the adequacy of the dispersion. Some investigators have commented that proper dispersion is more important than the method, equipment or technique used in conducting the analysis proper. If the powder is partly agglomerated, it will appear to be too coarse; whereas if the suspension is too concentrated, the coarse particles will tend to carry down the finer particles with them and a distorted sedimentation curve will be produced. It was found that approximately a 1.5% pulverized sugar suspension was optimum for this determination.

Production of a good dispersion of pulverized sugar in a suitable medium was of primary importance. Many different media have been employed for different powders although aqueous media are generally preferred owing to their ease of handling and evaporation. A suitable medium is one which (a) wets the sample readily but does not dissolve it, and (b) has a density and viscosity such that an analysis can be completed in a reasonable length of time. It is usual to add a low concentration of dispersing agent to prevent re-agglomeration or flocculation of the suspended material once the particles are dispersed. Unfortunately, the choice of a suitable medium and dispersing agent is a matter of trial and error, and many are very specific in their action.

In this investigation, a number of materials were tested for use as suspension media and dispersing agents. They are shown in Table I.

TABLE 1

Suspension media and dispersing agents tried in developing the proposed sedimentation method for particle size analysis

Suspension media	Dispersing agents
Methyl, ethyl, isopropyl, Butyl and octyl alcohols	Sodium pyrophosphate
Ether	Oronite D-40
Acetone	Oronite NI-W
Kerosene	Diallyl phthalate
Mineral oil	Aquawet
Glycerol	Sulfamic acid
Carbon tetrachloride	Sodium oleate
Benzene	Tween 20, 80
Propylene glycol	Span 20, 80, 85
	G-8916 P (Atlas Powder)
	Twitchell 8240

These were tried in various concentrations and combinations with pulverized sugar, and the resulting suspensions examined microscopically to see if complete dispersions resulted. The most suitable dispersion medium found for use in determining the particle size distribution of pulverized sugar consisted of isopropyl alcohol saturated with sucrose and containing 0.2% of Twichell 8240.

The results for a typical particle size distribution analysis, as determined by this sedimentation technique, on one type of pulverized sugar are shown in Table 2.

Another way of indicating these results is shown in Table 3.

Excellent reproducibility of the method is demonstrated in Table 4 where particle size determinations were made on one sample on 4 successive days.

The particle size distribution of pulverized sugars containing up to 8% starch, as well as starch-free samples, have been successfully analyzed.

TABLE 2

Particle size distribution data for one type of pulverized sugar

Height of fall	Time of fall	Calculated fraction size	Residue recovered	Finer than calculated fraction size
(h) cm.	*(t) min.*	*(d) microns*	*mg.*	%
21.0	0	Sample	111.1	100.0
20.7	5	56	103.6	93.2
20.3	10	40	93.6	84.2
19.9	15	32	87.0	78.3
19.5	22	26	79.8	71.8
19.1	35	21	74.2	66.8
18.7	60	16	47.7	42.9

TABLE 3

Particle size distribution for one type of pulverized sugar

Particle size range (microns)	Per cent distribution
over 56	6.8
56 to 40	9.0
40 to 32	5.9
32 to 26	6.5
26 to 21	5.0
21 to 16	23.9
under 16	42.9

TABLE 4

Reproducibility of method for one type of pulverized sugar

Particle size range (microns)	Per cent distribution for determination			
	No. 1	No. 2	No. 3	No. 4
over 40	3.1	4.2	4.4	4.6
40 to 32	3.6	3.7	3.2	2.7
32 to 26	3.9	3.5	4.2	3.3
26 to 21	4.6	5.9	6.8	5.9
under 21	84.8	82.7	81.4	83.5

Accurate calibration of the method is very difficult, particularly if standard pulverized sugar samples of known particle size distribution are not available. However, a one point check was made between the material passing through a 325 mesh screen (44 μ) and the results obtained by the proposed sedimentation method. Excellent comparisons were obtained and are shown in Table 5.

TABLE 5

Comparison of the screen and sedimentation methods on various pulverized sugars

Test sugars	1	2	3	4	5	6	7	8	9	10
By screening % under 44 microns	85.6	84.0	86.2	85.2	84.0	86.2	88.0	87.6	74.9	97.8
By sedimentation % under 40 microns	85.6	82.8	83.9	84.2	81.9	84.5	90.7	84.5	74.5	97.0

Another attempt at calibration was made by taking photomicrographs of isolated fractions after different elapsed settling times. The size of the largest particles in any one fraction were found to be very close to the calculated figures. It indicated that the sedimentation method was quite accurate down to 16 μ.

SUMMARY AND CONCLUSIONS

The Andreasen pipette sedimentation method for determining particle size distribution in the sub-sieve range has been adapted for use on pulverized sugars. The critical dispersion medium developed for this method consists of isopropyl alcohol saturated with sucrose and containing 0.2% of a dispersing agent (Twitchell 8240).

The procedure described gives accurate and reproducible results when determining the particle size distribution of pulverized sugar in the 16 micron diameter and larger size range.

Starch-free pulverized sugars, as well as pulverized sugars containing up to 8% starch, have been successfully analyzed.

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EXPLORATORY RAT AND CHICK BIOASSAYS OF SCALES FROM OCEAN PERCH AND HERRING AS ANIMAL FEED[a]

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Fish scales are a by-product of the fish packing industry and frequently create a waste disposal problem. The scales of some species are utilized for the extraction of pearl essence or the manufacture of fire-blanketing foams, but these uses are of small significance, and, in the case of the pearl essence extraction, fail to solve the disposal problem since only the guanine on the scales is used in this operation. In fish filleting plants, scales are removed from the fish by mechanical scalers and are washed away by jets of water, often finding their way into harbors where they settle to the bottom. They are not readily decomposed in water, and can accumulate to give rise to a state of pollution with attending problems of harbor clogging and production of objectionable odors.

The possibility of drying scales to manufacture them into feed for poultry, similarly to other fish wastes, has, of course, been given consideration by the industry in the past. Among the reasons cited for the common rejection of this practice, are: 1) the undesirable drying characteristics of fish scales, which tend to adhere to the walls of fish meal drying tunnels, 2) the abrasiveness of the dried scales which causes excessive wear on the grinding machinery, and 3) the lack of knowledge pertaining to the nutritive value of fish scales as animal feed.

It was the purpose of this investigation to determine whether or not a practical means could be found of utilizing fish scales in the manufacture of animal feed and to determine whether the resulting product could be fed to animals. Scales of the ocean perch *(Sebastes marinus)* and the Atlantic herring *(Clupea harengus)* were used in this research.

EXPERIMENTAL

In order to overcome the above-mentioned objections regarding the behavior of fish scales in drying and grinding operations, 2 possible methods of altering the undesirable physical properties of fish scales were considered. The first involved the breakdown of scales by chemical or enzymatic hydrolysis to yield a sludge capable of being dried and

[a] Contribution No. 1125 of the University of Massachusetts College of Agriculture Experiment Station, Amherst, Mass. Based upon a thesis submitted by N. I. Lemack in partial fulfillment of the requirements for the M.S. degree. The research reported in this paper was conducted by the University of Massachusetts under a contract with the U. S. Fish and Wildlife Service. It was financed by funds made available under provisions of Public Law 466, 83rd Congress, approved July 1, 1954, generally termed the Saltonstall-Kennedy Act.

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[d] Retired, present address: 52 Fearing Street, Amherst, Mass.

ground into a powder, and the second, the admixture of scales with other fish wastes and reduction to meal by conventional means.

Scales. Ocean perch scales discarded by filleting plants were collected in Gloucester, Mass. and dried the next day in Amherst, Mass. Herring scales, free of guanine, were collected and dried in a pearl essence plant located in Eastport, Maine, and shipped dry to Amherst. In both instances the drying temperatures were about 60° C.

Hydrolysis. Acid, alkaline and enzymatic hydrolyses of the scales were studied, the extent of hydrolysis being measured by reduction in insoluble solids content pursuant to treatment. Treatment with various concentrations of H_2SO_4 or NaOH, using a number

TABLE 1

Analysis of ocean perch scale hydrolysate, ocean perch scales, and herring scales used in feeding experiments

Constituent	Ocean Perch		Herring
	Hydrolysate	Scales	Scales
Moisture (%)	1.8	2.3	0.5
Protein (6.25 × % N)	34.0	49.3	71.6
Fat (% ether extract)	....	1.1	0.5
Ash (%)	60.1	47.3	27.4
Calcium (%)	10.5	14.4	9.0
Phosphorus (%)	5.5	7.9	5.1
Potassium (%)	0.17	....	0.07
Sodium (%)	1.2	....	.....
Sodium chloride (%)[1]	3.05	....	.....
Riboflavin (mg./100 g.)	0.177	....	.....
Cyanocobalamin (μg./g.)	0.002	0.044	0.043

[1] Calculated.

of different fish scale to water ratios, yielded from 47.6% hydrolysis (using NaOH to give a pH of 9.7, and a 1:50 fish scale to water ratio) to 57.8% hydrolysis (using 10% NaOH, with a 1:50 ratio) or 57.2% hydrolysis (when 5% H_2SO_4, with a 1:50 ratio, was used).

Hydrolysis of the scales in a 0.48% pepsin solution at a 10% fish scale solids level, and a pH of 1.0 to 2.0, gave 98% hydrolysis or better, after incubation for about 20 hours at 37° C.

Since the fish scale-water mixture had to be acidified prior to the pepsin hydrolysis, it became desirable to neutralize the hydrolyzate prior to drying it. Acidified or partially neutralized hydrolysate showed poor drying characteristics, and possessed a very strong and unpleasant odor. All hydrolyzates used in the feeding experiments were therefore fully neutralized with NaOH. They were dried down by first boiling off the bulk of the water by open kettle heating, and then removing the remainder of the water by drying in a dehydrator at about 60° C. The dried hydrolysate was ground in a hammer mill prior to use.*

Chemical and microbiological assays. Determinations for moisture, protein, fat, ash, calcium, phosphorus, potassium, sodium, riboflavin (Vitamin B_2) and cyanocobalamin (Vitamin B_{12}), were carried out by the appropriate chemical or microbiological methods described in the Official Methods of Analysis of the Association of Official Agricultural Chemists *(1)*.

Rat bioassays. Wistar strain albino rats, raised in the animal nutrition laboratories of the Food Technology Department, were used in these assays.

In the first series, 3 diets were used (Table 2). The control diet had casein as the sole source of protein; one experimental diet had neutralized, dried and ground ocean perch hydrolysate as the sole source of protein; and the second experimental diet had

* Further details on hydrolysis experiments are given in a report on "Chemical and Enzymatic Hydrolysis of Fish Scales," published in *Commercial Fisheries Review, 20*, No. 8, 1–3 (1958).

TABLE 2

Design of experimental rations for rat feeding tests

Component	First Bioassay			Second Bioassay			Third Bioassay		
	Control	Scale Hydrolyzate	Ground Scales	Control	Fish Meal/Ocean Perch 70:30	Fish Meal/Herring 70:30	Control	Fish Meal/Ocean Perch 90:10	Fish Meal/Herring 90:10
	(grams)	*(grams)*	*(grams)*	*(grams)*	*(grams)*	*(grams)*	*(grams)*	*(grams)*	*(grams)*
Casein	204.3	.....	.....	.....	.....	.....	.....	.....	.....
Ground dried ocean perch scales	.....	.....	510.75	.....	125.1	.....	.....	69.48	.....
Dried ocean perch scale hydrolysate	.....	600.87	.....	.....	.....	.....	.....	.....	.....
Ground dried herring scales	.....	.....	.....	.....	.....	87.6	.....	.....	52.23
Fish meal (60.3% protein)	.....	.....	.....	.....	.....	.....	564.49	508.01	508.01
Fish meal (61% protein)	.....	.....	.....	335.0	234.5	234.5	.....	.....	.....
"Protein Free" diet [1]	2015.7	1619.13	1709.25	1885.0	1860.4	1897.9	2765.51	2752.51	2769.76
Vitamin diet fortification mixture [1]	50.0	50.0	50.0	50.0	50.0	50.0	75.0	75.0	75.0
Calculated protein (%)	9	9	9	9	9	9	10	10	10

[1] Manufactured by Nutritional Biochemicals Corporation, Cleveland 28, Ohio.

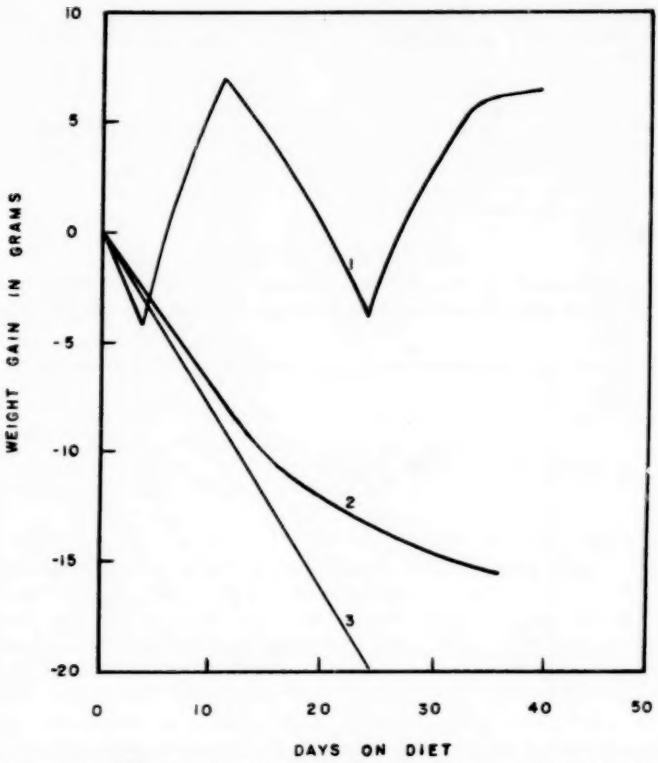

Figure 1. Weight changes in white albino rats fed dried ocean perch scales (curve 2), dried, pepsin-hydrolyzed, ocean perch scales (curve 3), and control diet (curve 1).

dried, ground (untreated) ocean perch scales as the sole source of protein. All diets were designed to be nutritionally complete (except for protein in experimental diets) and isocaloric, with a calculated protein level of 9%. Three groups of 14 rats each were used in this assay which was conducted for 42 days. Biological evaluation of these diets was carried out according to the paired-feeding technique of Mitchell and Beadles *(2)*. Results of this assay are shown in Figure 1.

In the 2nd series of rat tests, the control diet consisted of commercial steam-dried fish meal [1] as the sole source of protein; the experimental diets contained fish meal to the extent of 70% of the protein supply, with either ocean perch or herring scales contributing the remaining 30%. All diets were devised to be nutritionally complete (except for protein in experimental diets) and isocaloric, and provided a protein level of 10%. Three groups of 6 rats each were used in this assay which lasted 42 days. A 3rd series of tests was conducted using 90:10 fish meal-fish scale ratio. Results obtained in both these series are summarized graphically in Figure 2.

Chick bioassay. One-day-old male broiler chicks from a nearby commercial hatchery were fed 2 control and 4 experimental diets. Control diets consisted of 1) a ration in which soybean meal served as the main source of protein, and 2) a ration in which fish meal served as the main source of protein. The experimental diets comprised rations made up using the following approximate fish meal-fish scale ratios: 75:25, fish meal-

[1] Manufactured by the Gloucester By-Products Corp., Gloucester, Mass.

ocean perch scales (fish meal constituting 75% of the dietary protein, with ocean perch scales constituting the remaining 25%), 2) 50:50, fish meal-ocean perch scales, 3) 75:25, fish meal-herring scales, and 4) 50:50 fish meal-herring scales. All diets were nutritionally complete (except for protein in experimental diets) and had a calculated protein content of 15%. The exact composition of the rations is shown in Tables 3 and 4.

Replicate groups of 16 birds each were randomly assigned to treatment and were reared for the 3-week experimental period in electrically heated battery brooders with raised wire floors. Feed and water were supplied *ad libitum*. Chicks were weighed in groups, and weight gain was expressed in terms of average gain per chick. Resulting data are graphically presented in Figure 3.

RESULTS AND DISCUSSION

Results obtained in the first rat bioassay (Figure 1) indicate that both the hydrolysate and the raw ocean perch scales, as used, constituted inadequate

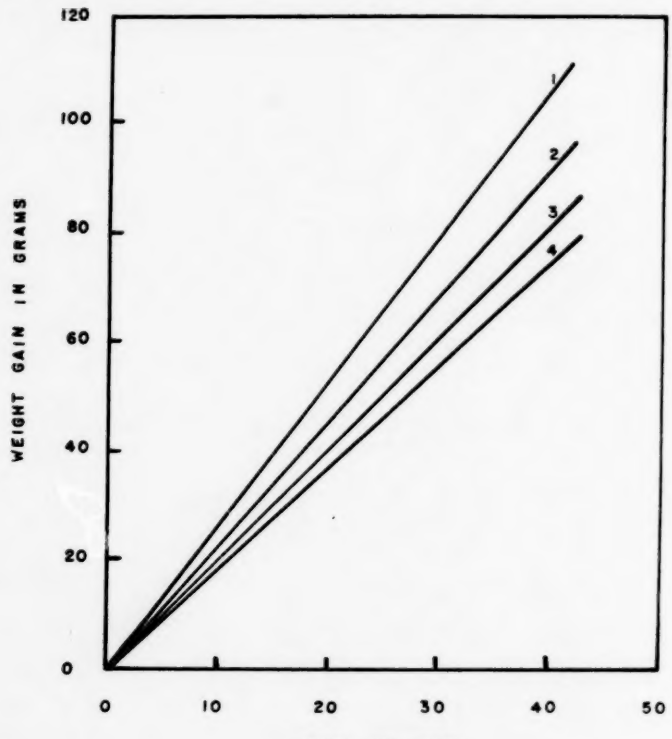

Figure 2. Weight changes in white albino rats on fish meal-fish scale diets:

Curve 1: Fish meal control for 10% fish scale diet
Fish meal-ocean perch scales (90:10)
Fish meal-herring scales (90:10)
Curve 2: Fish meal control for 30% fish scale diet
Curve 3: Fish meal-herring scales (70:30)
Curve 4: Fish meal-ocean perch scales (70:30)

TABLE 3

Basal diet premix for chick feeding tests

	Pounds
Cerelose [1]	52.0
Stabilized animal fat	2.0
Solka Floc [2]	1.0
Brewers dried yeast	3.0
Dried whey product	3.0
Corn distillers' dried solubles	3.0
Alfalfa meal (17%)	5.0
Calcite flour	0.7
Manganese sulfate (70%)	.03
Iodized salt	.1
Choline chloride-dry (25%)	.2
Dry vitamin A (10,000 I.U.)	.044
Dry vitamin D_3 (3,000 I.C.U.)	.018
Vitamin mix #1 [3]	.1
α-tocopheryl acetate	.009

[1] Commercial glucose.
[2] 99.5% pure cellulose, Mft. Brown and Co., Berlin, New Hampshire.
[3] Supplies per pound of ration: folacin 0.5 mgs.; riboflavin 2.0 mgs.; calcium pantothenate 2.0 mgs.; pyridoxine HCl 2.0 mgs.; menadione 2.0 mgs.; thiamine HCl 0.5 mgs.; biotin 25 μgs.; vitamin B_{12} 5 μgs.; niacin 20.0 mgs.; and procaine penicillin 5.0 mgs.

sources of protein for the sustenance of life. It will be noted that rats on both experimental diets never gained weight, but that rats on the control diet did gain weight, lost it, then gained it again. It may also be noted that the second weight gain in the controls coincides with the less severe losing of weight by the rats on the raw fish scale diet. These variations were due to the fact that amounts of the various rations for all the rats were kept equal. When the rats on the hydrolysate diet had all died, they were no longer restricting the supply of food to the rats on the other two diets. It was evident that the scales had been altered unfavorably in the course of hydrolysis, and therefore the hydrolysate was no longer considered worthy of further experimentation. This conclusion was, of course, supported by the results of total protein and Vitamin B_{12} analyses on the scales and hydrolysate.

TABLE 4

Design of experimental rations for chick feeding tests

Component	Soybean Control	Fish meal Control	Fish meal/ Ocean Perch 75:25	Fish meal/ Ocean Perch 50:50	Fish meal/ Herring 75:25	Fish meal/ Herring 50:50
	(lb.)	*(lb.)*	*(lb.)*	*(lb.)*	*(lb.)*	*(lb.)*
Basal diet premix	35	35	35	35	35	35
Cerelose	0.8	5.5	4.5	3.55	4.95	5.25
Dicalcium phosphate	1.6	....	0.45	0.9	0.45	0.9
Fish meal (60%)	....	9.5	7.15	4.75	7.15	4.75
Ocean perch scales	....	....	2.9	4.5	....	....
Herring scales	....	....	....	....	2.45	4.1
Soybean meal (44%)	12.4	....	....	0.75 [1]	....	....
Iodized salt	0.2	....	....	....	....	....
Calculated protein (%)	15	15	15	15	15	15
Kjeldahl protein (%)	15.2	16.3	15.5	15.5	16.5	16.2

[1] Added to bring protein content up to calculated level as supply of ocean perch scales had been exhausted.

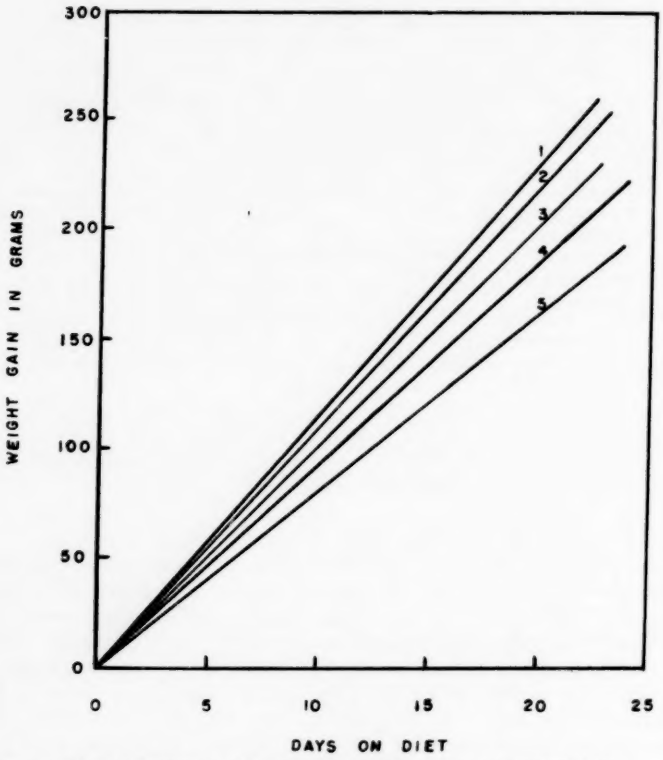

Figure 3. Weight changes in chicks on fish meal-fish scale, and control diets:

Curve 1: Fish meal-ocean perch scales (75:25)
Fish meal-herring scales (75:25)
Curve 2: Fish meal control
Curve 3: Soybean control
Curve 4: Fish meal-herring scales (50:50)
Curve 5: Fish meal-ocean perch scales (50:50)

Results obtained in the second rat bioassay (Figure 2) indicated that fish scales, either from ocean perch or from herring, when added to fish meal in quantities such as to represent 10% of the meal protein, under the experimental conditions used, showed no evidence of any adverse effects on the growth value of the meal. This is apparent from Figure 2 where it is seen that a single line represents the weight gain of all the rats on three diets in this assay.

The third rat bioassay was designed to investigate the effect of increasing the fish scales content of the fish meal. It is noted (Figure 2) that addition of herring scales to fish meal, such as to comprise 30% of the meal protein, seemed to have a slight adverse effect on the nutritive value of the meal, while a similar addition of ocean perch scales showed a still greater effect. Statistical analysis by Student's "t" test *(3)*, however, showed that there was

no significant difference (at the 5% level) between the weight gain of the controls and the rats on the 70:30, fish meal: herring scale diet. It did indicate a significant difference between the controls and the rats on the 70:30, fish meal: ocean perch scale diet, in favor of the control diet. Since the second bioassay had shown that ocean perch scales could be added to the extent of 10% of the meal protein, without adverse effect, it would appear that the maximum amount of ocean perch scales which might safely be added to fish meal, under the conditions studied, lies somewhere between 10% and 30% of the meal protein. The better performance of the herring scales as compared to the ocean perch scales (Figure 2) is probably related to the higher "protein" content of the former (Table 1).

Results of the chick bioassays, illustrated in Figure 3, were also treated by statistical analysis, using an analysis of variance and Tukey's test of significance of difference between any two means *(4)*. It was determined that there was no significant difference (at the 5% level) between the growth rates of the chicks on the control diets, and those of the chicks on either the 75:25 fish meal-ocean perch scale diet or the 75:25 fish meal-herring scale diet. There was, however, a significant difference between the rates of growth of the animals on the control diets and those on either of the 50:50 fish meal-fish scale rations.

CONCLUSIONS

Neither the dried, untreated, ocean perch scales nor the dried, pepsin-hydrolyzed, ocean perch scales proved capable of sustaining life in white albino rats. When used in combination with commercial fish meal, with the fish scales contributing 10% of the protein in the diet, either ocean perch scales or herring scales could be used without adversely affecting the growth rates of the rats. When used at the level of 30% of the ration protein, herring scales were found to show no adverse effect, while ocean perch scale did reduce the growth rates of the rats on the experimental diet. Thus, under the experimental conditions used, the maximum safe level of addition of fish scales to fish meal seemed to lie between 10% and 30% of the diet protein for ocean perch scales, and at or above 30% for herring scales.

When fed to one-day-old chicks, neither ocean perch nor herring scales, used in combination with fish meal to the extent of 25% of the protein content of the diet, showed any adverse effect on the growth rate of the test animals to three weeks.

Replacing 50% of fish meal protein with 50% of either ocean perch or herring scale protein markedly reduced growth. Additional studies designed to include a measure of the effect of fish scales on feed efficiency would be desirable.

Results of these studies appear to indicate that, since the scales constitute only about 1% of the weight of ocean perch, and heads, bones and tails constitute about 75% of the weight of the fish, the inclusion of scales in the other wastes in the proportion in which they naturally occur prior to the reduction of the wastes to fish meal, would not result in a lowering of the growth value of the resulting product for animal feed usage. In the case of waste herring scales from pearl essence operations, the addition of scales to other herring wastes might also be feasible, providing careful proportioning is practiced.

Further feeding tests and the approval of federal and state regulatory agencies concerned with feed control would certainly be required before any such addition should be undertaken in commercial practice.

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ON THE ACTION OF PURIFIED "TOMATO POLYGALACTURONASE"

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All of the recent investigations of "tomato polygalacturonase" were done with fairly crude preparations *(4, 6)*. Such studies have shown that these enzyme preparations cause a random hydrolysis of pectic acid to D-galacturonic acid. If the initial reaction velocity with pectic acid is taken as 100, those with tetra-, tri- and digalacturonic acids are, respectively, about 7.0, 1.58 and 1.05 *(4)*. "Tomato PG" has been reported to have an optimum pH of 4.5 with pectic acid as the substrate *(5)*.

Tomato PG was purified, after extraction of tomato pulp with NaCl, by adsorption on calcium phosphate gel followed by elution and fractionation of the eluate with $(NH_4)_2SO_4$ between 0.5 and 0.9 saturation *(8)*. The purified, water soluble enzyme had a specific activity of 5.8×10^{-3} PG u per mg. protein (1 unit of activity releases 1 mM of reducing groups per min. at pH 5.0 from a 0.5% pectic acid solution) and still carried out a random type hydrolosis of pectic acid to D-galacturonic acid.

Since yeast endo-polygalacturonase, which has been shown to be a single enzyme *(9)*, has an optimum pH of 4.5 on pectic acid, but that of 3.5 when acting on tetragalacturonic acid *(2)*, the optimum pH of tomato PG was determined on various oligo-uronides. It was found that the hydrolysis rates of tri- and tetragalacturonic acids exhibited two peaks at pH 3.5 and at 4.5. The hydrolysis of digalacturonic acid showed a single optimum at pH 4.5. Paper chromatographic analysis of the hydrolysis products of tetragalacturonic acid at pH 3.0, 3.5, 4.5 and 5.0 showed similar patterns, indicating that the type of activities at the two pH optima are similar. There is a preferred $(3 + 1)$ split during the very early stages of enzymic hydrolysis. Splitting of the central bond of tetragalacturonic acid, however, soon becomes evident and occurs at a somewhat slower rate than the $(3 + 1)$ cleavage.

The activity of tomato PG was also tested on oligouronides in which the terminal galacturonic acid unit was reduced to L-galactonolactone or oxidized to mucic acid lactone. When the substrate was either one of the two derivatives of tetragalacturonic acid, the initial reaction consisted solely of a cleavage of the central bond. The rate was roughly the same at pH 3.5 and at pH 4.5, but was slower than the rate of hydrolysis of tetragalacturonic acid. The two modifications of trigalacturonic acid were attacked much more slowly than the modified tetramers and only at the linkage farthest removed from the modified end-group. The digalacturonic acid derivatives were not attacked. From these experiments it was concluded that tomato PG hydrolyzes oligouronides preferentially at the first glycosidic bond adjacent to the reducing end-group. The central bond is hydrolyzed more slowly, and hydrolysis at the bond adjacent to the non-reducing end is so slow that it is obscured by the more rapid reactions at bonds 1 and 2. An end-group splitting α-galacturonidase is apparently not present since the modified dimers were immune to attack. Such an enzyme has been found in mold polygalacturonase preparations *(1, 7)*. Attempts to separate or

enrich one of the activities with optima at pH 3.5 or 4.5 were not successful. When the ratio of the *activity at pH 3.5/ activity at pH 4.5* was determined in various fractions during the purification procedure, after heating, treatment with alkali, acid, or passage over ion exchange resin, the values varied between 1.10 and 1.29 without showing a trend of separating the two activities.

In view of the double pH optima observed with oligouronides, tomato PG was also tested on an acid soluble pectic acid *(7)* with an average degree of polymerization of 14. An optimum pH at 4.5, reported by McColloch and Kertesz *(5)*, was confirmed, but in addition a second optimum at pH 2.5 was discovered. Such experiments are not possible with pectate of high molecular weight (as used by previous workers), since it precipitates at low pH values. The overall results indicate that the tomato PG preparation used contains at least two endo- PG components which are very similar in their properties but distinct from the previously described endopolygalacturonase from yeast *(3)*. The hypothesis best supported by the experimental evidence indicates that one enzyme hydrolyzes pectic acid by a random mechanism at pH 4.5 to galacturonic acid. The other enzyme also catalyzes random splitting of pectic acid, presumably at an optimum pH of 2.5, but it hydrolyzes the oligouronides at an optimum pH of 3.5. This enzyme cannot attack digalacturonic acid. Full details of our work will be reported in a publication to follow.

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